PRODUCTION OF NIACIN DEFICIENCY IN RATS

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Krehl and coworkers ('45a) have reported that rats fed a low-protein diet containing 40% corn developed a growth depression which was correctable by either niacin or tryptophane. These findings have been confirmed by Dann ('46), Singal et al. ('46), and Spector and Mitchell ('46).

Tryptophane and niacin are apparently interchangeably active in reversing growth retardations produced by certain diets in chicks (Briggs, '45), in rabbits (Wooley, '47), and in dogs (Hundley, '46).

Dann ('46) has shown that the corn grits diet of Krehl et al. ('45b) will sharply reduce the urinary output of N^1-methylnicotinamide (NMN) in the rat. It has been reported that the addition of tryptophane to various diets will cause a large increase in the urinary excretion of nicotinic acid and its derivatives in rats (Rosen et al., '46; Singal et al., '46), in horses and cotton rats (Schweigert et al., '47), and in man (Sarett and Goldsmith, '47; Perlzweig et al., '47).

These reports seem to indicate that tryptophane is an important precursor of niacin synthesis and that a niacin deficiency is produced when the diet contains insufficient tryptophane to compensate for the lack of preformed niacin.

However, Woolley ('46b) has prepared a concentrate from corn which has a "pellagragenic" action in mice. In addition he has reported ('46a) that either niacin or tryptophane will

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reverse the biological effects of 3 acetylpyridine. Kodicek et al. ('46) have reported that indole-3-acetic acid will produce the same growth depression in rats as will corn itself.

Groschke and Briggs ('46) and Krehl et al. ('46b) have found that certain amino acids have a marked growth-inhibiting action which can be overcome by niacin.

The present paper presents a study of the production of niacin deficiency in rats using diets free of any known "anti" factor, in which the only apparent etiologic agent was a relative lack of tryptophane. This has been possible by feeding a low percentage of casein supplemented with cystine or other tryptophane-deficient protein material.

METHODS

Male rats weighing 35 to 40 gm were placed individually in wire mesh cages at weaning and distributed to form groups according to weight and litter.

The diets used were modeled after the basal diet of Krehl et al. ('45) and consisted of casein (vitamin free) 15, sucrose 78, corn oil 3, salts (Osborne and Mendel) 4, and l(—)cystine 0.15 gm. Vitamins were incorporated in the ration in the following amounts: thiamine 0.2, riboflavin 0.3, pyridoxine 0.25, calcium pantothenate 2, choline 100, inositol 10, 2-methyl naphthoquinone 0.1, biotin 0.01 and pteroyl glutamic acid 0.1 mg %. The niacin content of these diets was practically zero and niacin was added only when indicated. The casein or other protein material was varied from 6 to 40% at the expense of the sucrose. l(—)cystine was added to most of the diets so that the cystine-methionine deficiency of casein was at least partially corrected, making tryptophane the principal amino acid limiting growth (Mitchell and Block, '46).

Growth periods varied from 3 to 8 or more weeks. Niacin tissue assays were done using *L. arabinosus* with a slight modification of the Snell and Wright ('41) method. Urines were collected under toluene and the N^1-methylnicotinamide estimated promptly using the method of Huff et al. ('45).

RESULTS

I. Effect of niacin at various levels of casein (plus cystine)

The growth of rats on diets containing 6 to 40% casein was compared at each level of casein with and without added niacin. l(—)cystine 0.15% was added to all diets.

Representative data are shown in table 1. The addition of niacin caused growth stimulation at each level of casein up to 20%. Statistical analysis however showed that only the niacin growth stimulation in 12% casein diets was certainly significant, there being less than 1 chance in 100 that the growth stimulation was accidental.

TABLE 1

Growth and niacin tissue levels as influenced by diet and supplemental niacin.

DIET [1]		GROWTH			NIACIN CONTENT OF TISSUES [3]		
Casein	Niacin	No. rats	Av. gain	t value [2]	No. rats	Liver	Muscle
%	*mg %*		*gm/wk.*			*μg/gm*	*μg/gm*
6	0	5	1.1				
6	2	5	1.9	0.95			
9	0	9	10.1		5	128 (115–155)	56 (39–73)
9	2	9	13.7	1.75	5	133 (110–157)	82 (75–94)
12	0	10	15.4		5	140 (120–173)	49 (40–70)
12	2	10	21.3	2.91	5	175 (135–212)	84 (79–93)
15	0	5	23.9		4	186 (165–200)	59 (58–60)
15	2	5	30.9	2.02			
20	0	9	28.8		4	172 (165–177)	82 (80–85)
20	2	9	30.0	0.70	4	181 (170–188)	84 (81–87)
30	0	3	26.9				
30	2	3	28.2	0.90			
40	0	4	29.7	0.37			
40	2	4	29.1				

[1] All diets included l(—)cystine 0.15%.

[2] Calculated according to Fisher's "Statistical Methods for Research Workers." The author is indebted to W. M. Gafafer for these analyses.

[3] The author is indebted to Dr. J. G. Wooley for these assays. Values within parentheses indicate range.

In other experiments rats have been kept on these diets for 4 weeks with growth results comparable to those shown in table 1. At the end of this period the diets were reversed at each casein level so that rats not receiving niacin now received it, and vice versa. In the 9 and 12, but not in the 20% casein groups, growth shifts in favor of the niacin groups appeared.

In another experiment the effect of added tryptophane was found to be similar to that of added niacin. Using the 12% casein ration and 4-week growth periods, 4 rats grew at the rate of 13.9 gm/wk. When 10 mg % niacin was added to the same diet using 4 litter mates, growth was at the rate of 16.8 gm/wk. Four hundred mg % dl-tryptophane in 4 additional litter mates increased the growth rate to 17.1 gm/wk., but when both niacin and tryptophane in the above amounts were added to a fourth group of litter mates no growth stimulation was evident. The reason for the latter observation was not clear.

II. Liver and muscle niacin levels

A close correlation was apparent between the tissue niacin levels and the percentage of dietary protein (table 1). Using the rats receiving 20% casein as normal, the 9 and 12% casein rats showed a reduced concentration of tissue niacin. The addition of 2 mg % niacin to the diet brought all muscle levels to normal and brought the livers to normal in the 12% but not in the 9% groups. The same amount of niacin did not alter the levels significantly in the 20% casein group.

III. Urinary N^1-methylnicotinamide excretion

Normally rats appear to excrete a large part of their known urinary niacin derivatives in the methylated form (Huff and Perlzweig, '42; Rosen et al., '46), and since the amount of N^1-methylnicotinamide excretion bears a rather constant ratio to the amount of other niacin compounds, the excretion of this substance should give a good index of the total excretion of niacin.

The data (table 2) show a close correlation with the growth and tissue niacin assay results. If the excretion of the rats receiving 20% casein is taken as normal, then the 9 and 12% casein rats showed a marked reduction in N^1-methylnicotinamide. The addition of 2 mg % niacin caused a rise to approximately normal levels in the 9 and 12% rats but had no consistent effect at 20% or more casein. The addition of 100 or 400 mg % dl-tryptophane to the 9 and 12% rations caused a very large increase in the excretion of N^1-methylnicotinamide.

TABLE 2

N^1-methylnicotinamide (NMN) excretion.

DIET [1]			NO. OF OBSERVATIONS	NMN EXCRETION PER 24 HOURS	
Casein	Added			μg/100 gm body weight	Range
	Niacin	dl-Tryptophane			
%	mg %	mg %			
9	0	0	4	29	(19–55)
9	2	0	4	204	(128–315)
9	0	100	4	429	(189–633)
12	0	0	27	29	(6–75)
12	2	0	15	127	(16–360)
12	10	0	11	174	(100–268)
12	0	100	4	444	(277–607)
12	0	400	16	1074	(300–1980)
12	10	400	8	945	(365–1880)
20	0	0	5	121	(27–371)
20	2	0	6	112	(20–172)
30	0	0	4	90	(27–120)
30	2	0	5	97	(31–126)
40	0	0	4	195	(26–504)
40	2	0	4	223	(117–410)
Stock [2]	0	0	4	431	(246–828)

[1] All diets contained added l(—)cystine 0.15%.

[2] Ground Purina Dog Checkers.

IV. *Other tryptophane deficient diets*

The diets mentioned thus far seem to produce only a mild deficiency as judged by growth. After 8 to 10 weeks on the 9

or 12% rations, the response to niacin is no longer consistent. Also there is considerable litter variation and controls must be used in each case. Therefore these diets are not entirely suitable for routine use in studying niacin deficiency.

Other diets were constructed in which the relative deficiency of tryptophane was greater. In each instance rats on these diets as shown in table 3 showed a marked depression of

TABLE 3

Diets for production of moderate to severe niacin deficiency in rats.

CASEIN	TRYPTOPHANE DEFICIENT PROTEIN	TOTAL PROTEIN GM %	NIACIN MG %	ADDED DL TRYPTOPHANE MG %	TOTAL TRYPTOPHANE IN DIET MG %	GM/WK. GAIN	NMN [1] EXC. µG/DAY
9 gm %	+ 9 Ess. Am. Ac.[2] 7.9 gm %	16.9	0	0	108	2	7
9	+ "	16.9	10	0	108	18	—
9	+ 10 Ess. Am. Ac. 8.1 gm %	17.1	10	200	308	17.5	782
9	+ Acid hydrolyzed Casein 4 gm %	13	0	0	108	6	
9	+ "	13	10	0	108	20	
9	+ Oxidized casein[3] 5.4 gm %	14.4	0	0	108	3.5	
9	+ "	14.4	10	0	108	18	
9	+ Gelatin 3 gm % and l(—)cystine 0.15%	12	0	0	108	3.5	7
9	+ "	12	10	0	108	17.5	354
9	+ "	12	0	75	183	18.2	447
9	+ " 5.4 gm %	14.4	0	0	108	4	
9	+ " "	14.4	10	0	108	23	
9	+ " 9 gm %	18	0	0	108	1.8	10
9	+ " "	18	10	0	108	15.4	306
10	+ Gelatin + Am. Ac. 10[4]	20	0	0	120	2	
10	+ "	20	2	0	120	25	
10	+ " plus tryptophane	20.2	0	200	320	31	

[1] N¹-methylnicotinamide.

[2] Essential amino acids added in same amounts as given by Rose ('38) for meeting minimum requirements of rats.

[3] Prepared according to Toennies ('42).

[4] Amino acids added so as to be equivalent of 20% casein with amino acids except tryptophane.

growth which was correctable by either tryptophane or niacin. The use of tryptophane and niacin together did not increase the growth response over that when either one was used alone in optimum amounts.

DISCUSSION

From the data presented it seems clear that when growing rats are maintained on a ration in which tryptophane seems to be the chief amino acid limiting growth, a marked diminution in niacin synthesis occurs with a resultant niacin tissue deprivation and depressed growth. When tryptophane is added to the diet, either as casein or tryptophane itself, an increase in niacin synthesis occurs.

These facts strongly indicate that tryptophane is the dietary precursor of niacin. Similar conclusions have been published by Singal et al. ('46), Rosen et al. ('46) and Schweigert et al. ('47).

The action of niacin in promoting growth in the presence of an apparent deficiency of tryptophane has been explained by Krehl et al. ('46a) as being due to increased efficiency of utilization of tryptophane. A similar conclusion was reached by Spector and Mitchell ('46) as the result of a paired feeding study.

However, it is also possible, and seems more likely to the writer, that the niacin acts to "spare" tryptophane. Since tryptophane seems to be the dietary precursor of niacin, a certain portion of it must be used up in this process. If niacin is supplied preformed, the tryptophane may be spared of this function, making it available for tissue building. Some evidence in this direction is noted in table 3 since with increasing amounts of casein and a constant amount of niacin, there was no notable increase in excretion of N^1-methylnicotinamide (NMN). On the other hand, without niacin in the diet the NMN excretion varied roughly with the per cent of casein at least up to the level where growth was independent of the niacin intake. This observation could be explained by assuming that with adequate preformed niacin in the diet, the trypto-

phane is metabolized along with the other amino acids in a manner not leading to niacin synthesis. However, when a large amount of tryptophane is added, more than can be used in tissue building because of the limited supply of other amino acids, niacin is formed in excess even in the presence of adequate dietary niacin.

Since the diets used in this study were probably free of any "toxic" factors (with the exception of the gelatin diets) it seems evident that the primary etiology in the production of a niacin deficiency in rats is not an "anti" or "toxic" substance. If such factors have any role at all it must be only a contributory one. It is to be noted that in the report by Kodicek et al. ('46) on the use of indole-3-acetic acid, the basal diet used was 10.5% casein plus 0.15% cystine, a diet which by itself would likely produce a mild niacin deficiency according to the data reported in this paper.

CONCLUSIONS

Growing rats, maintained on a basic low-casein diet in which tryptophane was the chief limiting amino acid, developed a condition characterized by growth depression, diminished tissue niacin levels, and diminished excretion of N^1-methylnicotinamide.

This condition was interchangeably corrected by either tryptophane or niacin.

Deficient rats treated with tryptophane showed a marked increase in niacin synthesis.

The possible mechanisms involved are discussed.

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A BIOASSAY FOR PROTEINS AND PROTEIN DIGESTS

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THREE FIGURES

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In the course of large scale preparation of enzymatic digests of lactalbumin,[3] it was necessary to investigate the nutritional quality of the product for evaluation of its probable usefulness in human nutrition and as a routine control of the manufacturing procedure.

A method of rat bio-assay was devised which requires only 2 weeks to obtain a result, requires a minimum of chemical analyses, satisfies the dictum that accuracy of bio-assays is increased by comparing test samples with a standard substance and simulates the conditions of the greatest usefulness of protein digests in human nutrition, i.e., tissue regeneration after a period of negative nitrogen balance.

Casein was chosen as the standard to which other proteins or protein hydrolysates were compared as it is readily obtainable, inexpensive, and amino acid analyses of different samples have shown remarkably good agreement (Dunn et al., '46). Vitamin test casein GBI [4] was the particular brand used.

The basis of the assay is the comparison of the amount of dietary nitrogen in the form of casein with the amount of

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[2] Present address: Wyeth Inc., Nutritional Division, Mason, Michigan.
[3] Lactamin, Wyeth, Incorporated, Philadelphia, Pa.
[4] General Biochemicals, Inc., Chagrin Falls, Ohio, 14.3% nitrogen.

other forms of dietary nitrogen per 24 hours required to maintain an adult rat for 1 week with no loss or gain in body weight, after a preliminary period of 1 week on a protein-free diet, caloric intake being held constant. The nutritional quality of the nitrogen source being tested is then numerically expressed by the ratio of the weight maintenance level of casein nitrogen over that of the nitrogen source being tested, casein nitrogen being assigned a nutritional index of unity.

EXPERIMENTAL

Test procedure

After completion of preliminary experiments the procedure adopted was that described in the following paragraphs.

Seventy or more male Wistar rats of approximately the same age, weight and previous history were selected for assay groups. The weight varied from 180 to 300 gm. In any one test the variation between the weights of the rats was within 50 gm.

The rats were individually caged in a constant temperature room maintained at 23° ± 1°C. Weighed amounts of food were fed in containers which could not be easily upset. Any spilled food was recovered, weighed, and an equivalent amount given to the rat at the next feeding. Any rat that persistently spilled his food was excluded. Water was supplied *ad libitum*. Separate supplements of 25 μg thiamine hydrochloride, 40 μg riboflavin, 25 μg pyridoxine, 100 μg calcium pantothenate and 20 mg of choline chloride were given daily and 1 mg of α-tocopherol and 0.3 mg of vitamin K once weekly. The feeding and weighing of the rats were done at the same hour every day.

The assay interval consisted of 2 periods — the depletion period and the test period — each of 1 week's duration. The daily allowance of ration, hence caloric and nitrogen intake, was fed in amounts proportional to the average surface area of the test animals at the start of the assay. The amount of

the diet to be fed was calculated to the nearest gram on the basis of 2.35 gm (11 cal.) per square decimeter of surface area. Surface area = 11.36 × weight $^{2/3}$ (Carman and Mitchell, '26).

The diet fed during the 7-day depletion period had the following composition: cerelose 79, hydrogenated cottonseed oil [5] 15, salt mixture 4 (U.S.P. no. 2) and cod liver oil U.S.P. On the eighth day, the rats received the usual amount of protein-free diet at 9 a.m. At 11.00 a.m. the rats were weighed and at the same time any uneaten food removed from the cage. This procedure was found to give the most consistent weights. Some of the rats had eaten their ration within the first few hours on the previous day and consequently would have been in a fasting state unless fed on the following morning before the time of weighing. Without this additional feeding the weights of the rats varied considerably, depending on how rapidly the diet on the seventh day had been consumed. The weight loss with this procedure in 12 groups of 70–100 rats varied from 8.6% ± 2.1% of the original weight. In any single experiment the average deviation was ± 0.7%.

Sixty-five of the depleted animals, selected on the basis of conformity to the average weight drop, food habits in regard to food spillage and consumption of all the diet, were divided by weight into 13 groups of 5 each. With these 13 groups, 3 test substances and a casein standard could be compared.

One group of 5 was continued on the protein-free diet. Three groups of 5 were fed diets containing 0.36, 0.72 and 1.08% casein nitrogen which is equivalent to 2.5, 5.0 and 7.5% casein. The remaining 9 groups received equivalent amounts of nitrogen obtained from each of the 3 protein materials being tested. These nitrogen levels were chosen because under the conditions of the experiment the weight change values obtained were found to fall in a suitable range for interpolation of the value of the amount of nitrogen fed daily which would cause neither loss or gain from the depleted weight.

[5] Crisco.

The dried test substances[6] were incorporated into the protein-free diet at the expense of the cerelose, so that all diets were very nearly iso-caloric, containing 47 cal. per gm of diet.

These diets were then fed on the eighth day at about 2 p.m., several hours being required to divide the rats into groups, and from then on for 6 more days. On the fifteenth day the animals were fed at 9:00 A.M and weighed finally at 11:00 A.M. The rats were returned to the stock diet, supplemented with whole milk powder, for 2 or 3 weeks before being used for assay.

A plot was made of the change in weight of each group from the end of the depletion period to the end of the test period against the milligrams of nitrogen fed daily. The amount of nitrogen of each protein or digest necessary to maintain a constant weight in the depleted rats was taken from the graph; this was the point at which the curves crossed the point of zero weight change. Nutritional indexes were then calculated.

Preliminary experiments

The weight changes of protein-depleted rats fed various levels of casein were investigated. Seven groups of 5 rats each (average weight, 220 gm) were depleted of protein for 1 week. One group was continued on the protein-free diet, the other 6 groups received 10 gm of diets containing respectively, 0.29, 0.57, 0.86, 1.15, 1.43 and 1.72% casein nitrogen. The groups were weighed daily for the next 12 days and the average daily weight change from the weight at the end of the depletion period was plotted against the daily nitrogen intake. The curves for several of the days are presented in figure 1. It may be seen that an approximately straight-line relationship was obtained in the regions of low nitrogen intake. The amount of daily nitrogen sufficient to maintain a constant weight was interpolated from each curve. Values obtained on the fifth to the twelfth days (excluding the tenth

[6] Hydrolysate solutions presented for assay were dried from the frozen state.

day) were 82, 85, 80, 78, 79, 80 and 81 mg of casein nitrogen (average 80.8) required per day for weight maintenance. Since a constant value was obtained from at least the fifth day on, it was decided that a 7-day test period would be of sufficient duration.

The validity of body weight changes, as a measure of protein nitrogen utilization under the conditions described in the above section, was investigated by a series of nitrogen balance studies patterned after those used by Melnick and

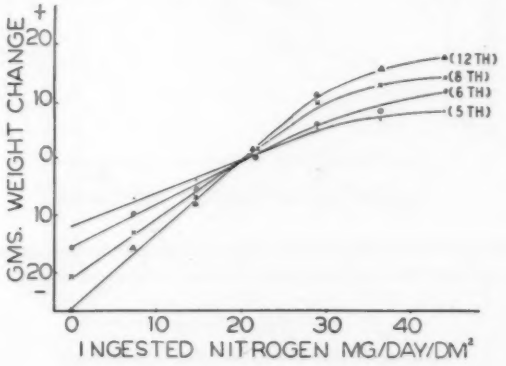

Fig. 1 The weight change of protein-depleted rats fed varying amounts of casein nitrogen. (The number in the parenthesis indicates the number of days of feeding.)

Cowgill ('37) in the determination of the minimal protein requirement for the establishment of nitrogen equilibrium in dogs. Rats from groups being fed casein diets were placed in metabolism cages and urine and feces were collected for the last 4 days of the assay test period. The average nitrogen balances for the 4-day periods and average changes in weight during the test period were plotted against daily nitrogen intake. The results of the typical experiment, presented in figure 2, show close positive correlation between nitrogen balance and body weight change with respect to daily nitrogen ingestion, hence it may be assumed that the weight changes observed are valid criteria of comparative nitrogen utiliza-

tion. In all experiments, the daily amount of nitrogen required for nitrogen balance equilibrium was less than necessary for weight maintenance.

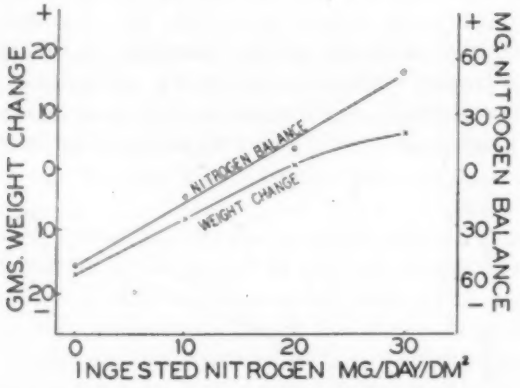

Fig. 2 The relationship of nitrogen balance and body weight change of protein depleted rats to ingested casein nitrogen. (Each point is an average of 5 values.)

TABLE 1

Assays of individual proteins and digests.

PROTEIN OR DIGEST	NUTRITIONAL INDEX
Casein (SMACO vitamin test)	1.00
Lactalbumin (Labco)	1.61 ± 0.08 [1] (6) [2]
Pancreatic digest of lactalbumin	1.44 ± 0.07 (13)
Crystalline Edestin	0.78, 0.73
Casein + 2% dl-methionine	1.65
Pepsin digest of liver protein	1.18
Equal parts of casein and lactalbumin	1.24

[1] Average deviation.

[2] Number of determinations in parentheses; otherwise 1 determination.

RESULTS

The assay procedure described was used routinely to evaluate the nutritional quality of commercial batches of pancreatic digests of lactalbumin. The digests were found to be of uniform nutritional quality (table 1). In table 1 is also pre-

sented a list of nutritional indexes found for several other proteins and protein digests by this method of assay.

The results of a typical assay are presented in figure 3. In this assay a sample of lactalbumin digest, undigested lactalbumin, and a sulfuric acid hydrolysate of casein were compared with the casein standard. The daily amounts of nitrogen required for weight maintenance in mg/day/dm² were casein 20.9, lactalbumin 12.6 and lactalbumin digest 15.8, respectively. The nutritional indices were calculated to be 1.58 for lactalbu-

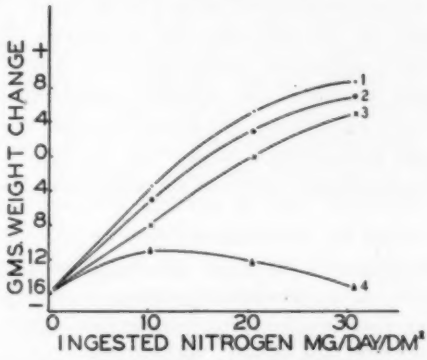

Fig. 3 Body weight change of protein-depleted rats when fed varying levels of casein, lactalbumin, pancreatic digest of lactalbumin and acid hydrolysate of casein. o — casein (curve 2); pancreatic digest of lactalbumin (curve 1); x — lactalbumin (curve 3); acid hydrolysate of casein (curve 4).

min and 1.39 for lactalbumin digest. In this and other tests the enzymatic digests of lactalbumin, either commercial or laboratory preparations, while always nutritionally superior to casein, were found to be inferior to undigested lactalbumin.

The weight change curves are typical of the results constantly obtained (higher nitrogen intake causes increase of weight) except in the case of the acid hydrolysate of casein. This product, fed in a small amount (approximately 10 mg N/dm²/day), does not cause a weight loss as large as does a diet entirely devoid of protein nitrogen; however increasing amounts of nitrogen lead to a progressively greater weight

loss. From these results it would seem possible that a large amount of protein hydrolysate nitrogen deficient in 1 or more amino acids would be more harmful to the animal than a diet entirely free of nitrogen. Such an effect has been reported by Frazier et al. ('47) who employed experimental diets composed of mixtures of crystalline amino acids.

In the course of this investigation 4 large groups of rats were maintained for assay purposes. Each group was used for 2 or 3 assays before being discarded. One group was subjected to 6 successive assays to determine if the increasing age and weight of the animals and repeated stresses of the procedure would affect the value obtained for the amount of casein nitrogen required for weight maintenance.

In table 2 are presented the experimental values obtained for casein and lactalbumin using the various assay groups. The weight maintenance level of nitrogen was not found to be

TABLE 2

Nitrogen requirement for weight maintenance of rats partially depleted of protein.

ASSAY GROUP	SUCCESSIVE ASSAYS	AVERAGE BODY WEIGHT AT DEPLETION	DAILY RATION ALLOWANCE	MG N/DAY/DM.[3]	
				Casein	Lactalbumin
		gm	*gm*		
I	1	189	10	21.2	...
	2	205	10	21.0	...
	3	221	11	20.9	12.6
II	1	175	10	25.0	...
	2	197	10	22.6	...
	3	210	10	24.1	13.3
	4	228	11	22.8	...
	5	236	12	20.1	...
	6	254	13	21.6	13.3
III	1	208	10	20.0	13.1
	2	224	11	20.1	...
IV	1	168	10	20.6	12.0
	2	228	11	20.2	12.9
Average				21.6 ± 1.1	12.9 ± 0.4

dependent on the group of rats used or to be affected by the number of times up to 6 that the group has been used. The average value of all the determinations was 21.6 ± 1.2 mg of casein nitrogen/day/dm². The average value for lactalbumin was 12.9 ± 0.4. The average deviation of approximately ± 5% indicates that under these experimental conditions consistent results may be obtained.

DISCUSSION

By subjection of the assay animals to a state of protein depletion, a condition is created which simulates that present in human patients requiring dietary protein therapy. Depletion of the rats' body stores of protein and the feeding of dietary protein at sub-optimal levels probably enhances the sensitivity of the assay, since the nutritional economy of the animals would require that the protein nitrogen be maximally utilized for the more essential processes of tissue maintenance. Under these conditions the responses produced by proteins or digests of different nutritional characteristics are emphasized. The response produced by each test sample is compared with that produced by a casein standard, thus neutralizing extraneous factors and allowing less exact specifications of the weight, age and breed of the rats employed. Since the response is evaluated with respect to the amount of nitrogen fed, the nutritional index is a measure of the digestion, absorption and utilization of the protein test substance.

Each group of rats may be used for several assays, provided a repletion period of 2 or 3 weeks is permitted between assays. The increasing size of the animal in subsequent assays is compensated for by increasing the daily dietary caloric and protein nitrogen allowance and by calculating the nitrogen maintenance requirement on the basis of surface area. The simplicity of the method recommends it for routine assay purposes. The 2-week assay period is relatively short for an animal assay. The only manipulations are those of weighing the rats and the daily rations; chemical analysis is limited

to that of the determination of nitrogen in casein and in the proteins or protein digests being tested.

SUMMARY

An assay for the evaluation of the nutritional quality of proteins and protein digests has been described. The assay is based on the determination under standardized conditions of the amount of fed protein or protein digest nitrogen necessary daily to maintain a constant weight in adult rats that have been partially depleted of body protein. Rats are fed a protein-free diet for 1 week; various groups are then fed varying levels of each test sample for the second week. The daily allowance of ration is proportional to the average surface area of the rats. By plotting body weight change during the second week against daily nitrogen intake, it is possible to interpolate the amount of nitrogen necessary to maintain constant weight in the partially depleted animal. Each test substance is evaluated by comparison with a casein standard.

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THE NUTRITION OF THE MOUSE

I. A DIFFERENCE IN THE RIBOFLAVIN REQUIREMENTS OF TWO HIGHLY INBRED STRAINS [1]

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ONE FIGURE

(Received for publication June 11, 1947)

The riboflavin requirements as well as the symptoms of riboflavin deficiency have been described for many species of laboratory animals. Bessey and Wolbach ('39) reported in some detail the cutaneous and ocular lesions shown by rats deficient in this vitamin. In the same year Street and Cowgill ('39) published results obtained with dogs maintained on a synthetic, riboflavin-deficient diet. These workers found that a daily supplement of 25 μg of riboflavin per kg of body weight would maintain dogs in apparent good health for a considerable period of time. Lippincott and Morris ('41–'42) and Morris and Robertson ('42–'43) studied many aspects of the problem using mice of the C_3H strain (highly susceptible to spontaneous mammary tumors). From a clinical viewpoint the monkey is a most interesting experimental animal. For this reason especially the work of Cooperman et al. ('45) deserves attention. These workers have concluded that the

[1] This work was supported by a grant from the American Cancer Society on recommendation of the Committee on Growth of the National Research Council. Some of the early experiments were carried out with funds from the Nutrition Foundation, Inc., and the Anna Fuller Fund.

monkey requires 25 to 30 μg of riboflavin per kg of body weight, figures in good agreement with those of Street and Cowgill ('39) for the dog. Cooperman et al. ('45) have described the cutaneous and ocular changes seen in the riboflavin-deficient monkey. They also reported lowered red and white cell counts and hemoglobin levels. Kornberg, Daft and Sebrell ('45) described similar blood changes in riboflavin-deficient rats. It is rather interesting to note that the blood picture of the monkey could be restored to normal more readily when riboflavin was administered together with whole liver, and that in the rat the red cell count could be restored with riboflavin but that the granulocytopenia responded best to folic acid. Finally, Bird and associates ('46) have determined the riboflavin requirement of chicks and poults to be, respectively, 2.75–3.25 and 3.25–3.75 μg per gm of food.

The mouse has long been known as an ideal laboratory animal for studies in genetics, cancer, infectious diseases, serology, etc. Yet by comparison with the rat very little is known of the specific nutritional requirements of the mouse. The need for quantitative information concerning the demand for riboflavin is enhanced by the possible relation of this vitamin (along with others, of course) to the cancer problem. The protective action of riboflavin against liver tumors induced by azo dyes has been demonstrated by Miller and associates ('41) and by Sugiura and Kensler ('41). While most of the azo dye work has been done with rats, due to the peculiar susceptibility of this species, one must consider the possibility that riboflavin may exert a similar protective action in the mouse, which is susceptible to o-amino-azotoluene as shown by Andervont and Edwards ('42–'43) and by Kirby ('45). Morris and Robertson ('42–'43) have studied the retarding effect of riboflavin-deficient diets on the growth of spontaneous mammary tumors.

In view of the need for precise information about the nutritional requirements of mice it was decided to study their rates of growth on several synthetic diets differing only in their content of riboflavin. Since Rogers, McElroy and Cowgill

('42) had noted the existence of strain differences in the nutritional requirements of mice, this investigation was carried out with 2 highly inbred strains — C_{57} (low incidence of spontaneous mammary tumors) and A (high tumor incidence).

EXPERIMENTAL

Five synthetic diets differing only in their riboflavin content were used in this study. Diet 120 had the following percentage composition: "vitamin-free" casein 30, hydrogenated cottonseed oil [2] 15, roughage [3] 3, salts [4] 7, dextrose C.P. 45, linoleic acid 0.41, and cod liver oil concentrate [5] 0.30. Each 100 gm of ration also contained the following vitamins: α-tocopherol 9 mg, choline 150 mg, inositol 100 mg, p-aminobenzoic acid 100 mg, calcium pantothenate 3 mg, nicotinic acid 1 mg, thiamine 0.5 mg, and pyridoxine 0.5 mg. While no riboflavin was added to this basal ration, it was found by fluorometric assay to contain as trace contamination 0.03 mg of this vitamin per 100 gm of diet. The other 4 rations used here differed from the basal in containing the following added riboflavin per 100 gm: no. 121 diet 0.2 mg, no. 122 diet 0.4 mg, no. 123 ration 0.6 mg, and the no. 124 diet 1.0 mg.

A total of 108 male mice of the C_{57} and A strains, weaned when 21 days old, were used in these studies. The animals were placed in individual screen-bottom cages and fed diet 120 ("riboflavin-free") for 1 week. At the end of this depletion period they were divided into 5 groups which were fed, respectively, diets 120, 121, 122, 123, and 124. Care was taken to distribute litter mates as uniformly as possible. The cages were kept in an air-conditioned room maintained at 75°F. and 50% relative humidity. The animals were weighed daily (with a few exceptions in the early phases of the experiment).

To determine the effect of riboflavin-low diets on adult mice, males of the C_{57} strain (16 to 18 weeks old, raised from

[2] Crisco.
[3] Ruffex.
[4] Sure's Salts no. 2.
[5] White's Cod Liver Oil Concentrate Liquid.

weaning on a synthetic diet high in riboflavin and similar in composition to no. 124) were placed on diets 120 and 121. These animals were weighed once a week.

In a number of instances food consumption was measured by placing 4.0 gm of the diet in a half-ounce glass jar into the cover of which a hole of ¾-inch diameter had been cut. A circular piece of ¼-inch mesh wire screen was laid loosely on top of the food. This piece was small enough to sink freely toward the bottom of the jar as the food was consumed. The entire jar assembly was placed in a 150-ml beaker. Usually in the course of 24 hours some food was spilled into the beaker and was weighed together with the residue in the cup. In another series of experiments it was found that food spillage outside the beaker was ordinarily so small as to be completely insignificant.

Red and white cell counts were done on blood obtained by clipping the tip of the tail; hemoglobin was determined by the alkaline hematin method with the aid of the Evelyn photoelectric colorimeter (Evelyn, '36). Riboflavin determinations on muscle and liver of 70 animals were carried out by the method of Peterson, Brady and Shaw ('43).

RESULTS

A distinct strain difference became apparent in the growth response to the riboflavin-free diet during the 1-week depletion period. Mice of the C_{57} strain gained weight steadily during this interval while A strain mice gained less weight and even showed some weight loss before the end of the depletion period. In the first group of experiments (fig. 1 A) maximal growth was obtained with the C_{57} strain when the diet contained 0.4 mg of riboflavin per 100 gm of diet (shown graphically as open circles). On the other hand the A strain showed maximal growth only at a riboflavin level of 0.6 mg per 100 gm of diet (open triangles). In figure 1 A the results with diets 123 and 124 (0.6 and 1.0 mg level) were combined since they were identical.

To test further the apparent strain difference in riboflavin requirement more mice of both strains were placed for 1 week on the riboflavin-free diet (no. 120) after which they were fed diets 122 or 123 (0.4 and 0.6 mg of riboflavin per 100 gm of diet, respectively). The results are shown graphically in figure 1 B. The response of the 2 strains to the depletion

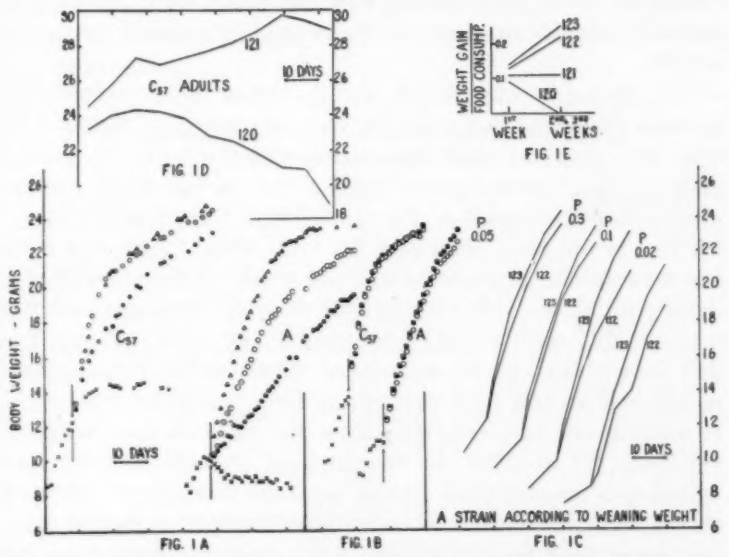

Fig. 1 Weights during the 7-day depletion period (the ends of which are indicated by arrows) are averages of all comparable groups regardless of subsequent riboflavin levels. The graphic symbols are as follows: diet 120 ×××; diet 121 ●●●; diet 122 ○○○; diets 123 and 124 combined △△△; diet 123 ■■■. P values were calculated on the basis of total weight gains above the weaning weights. Mice shown in figure 1 A were followed until 70 days old, those in figure 1 B until 50 days old.

diet was identical with that observed in the first experiments (fig. 1 A). Mice of the C_{57} strain again grew equally well on diets 122 and 123 while A strain animals grew better on the diet with the higher riboflavin level. In these experiments, however, the difference was small though significant as seen by the P value calculated for the respective increase in weight

from age 21 days to 50 days. To determine the reason for the large difference seen in figure 1 A and the small difference in figure 1 B shown by strain A on diets 122 and 123 these animals were grouped according to weaning weight (fig. 1 C). The animals fell into 4 groups: 7–7.9, 8–8.9, 9–9.9, 10 to 10.9 gm. In all 4 groups the animals receiving the higher riboflavin level grew better, but the difference is not statistically significant for the 2 groups of greatest weaning weight.

The efficiency with which the A strain mice utilized the various diets for body weight gain is shown in figure 1 E. The first point of each line represents the ratio of weight gain to food consumption during the initial week on the riboflavin-free depletion diet (no. 120). The final point on the line is the ratio computed for total weight gain and food consumption in the second and third weeks of the experiments during which the animals received diets of varying riboflavin content. The mice which were continued on the riboflavin-free diet showed during the second and third weeks of the experiments less weight gain per gm of food consumed (the ratio is actually slightly negative since the animals lost weight). On diets 122 and 123 the weight gain per gm of food consumed was greater than during the depletion period. Mice of the C_{57} strain showed a considerably greater weight gain per gm of food consumed during the depletion week than did animals of the A strain.

When adult male C_{57} mice were fed the riboflavin-free diet (no. 120), they continued to gain weight for a short time. Soon, however, they slowly declined in weight and gradually showed the lesions characteristic of riboflavin deficiency. During the eleventh week on this diet the weight loss became much more pronounced and during the tenth week the first death in this group occurred. Adult mice fed diet 121 continued to gain weight slowly. These results are plotted in figure 1 D.

The riboflavin content of muscle and liver is shown in table 1 together with red and white blood cell counts and hemoglobin levels. It is interesting to note that the A strain ani-

mals showed practically identical tissue riboflavin levels and blood pictures on diets 121, 122, and 123. On the other hand the C_{57} strain reared on diet 121 had distinctly lower tissue riboflavin levels than when raised on diet 123. The C_{57} mice on diet 121 also had lower red cell counts than did comparable animals on diets 122 and 123. The adult C_{57} mice maintained on diets 120 and 121 had the lowest red cell counts, hemoglobin levels and muscle and liver riboflavin concentrations of all the groups studied.

TABLE 1

Compilation of data on blood counts and the riboflavin content of muscle and liver.

STRAIN	DIET	CELL COUNTS		HEMOGLOBIN %	RIBOFLAVIN μG/GM WET TISSUE	
		RBC $\times 10^6$	WBC $\times 10^3$		muscle	liver
C_{57}: young mice	121	9.35(5)[1]	10.2(4)	17.4(4)	3.5(5)	25(4)
	122	9.97(13)	9.3(12)	16.9(13)	4.4(14)	30(14)
	123	10.02(11)	7.3(11)	17.1(11)	5.3(11)	32(11)
A: young mice	121	9.23(6)	6.7(6)	15.1(6)	6.0(7)	33(6)
	122	8.98(19)	6.6(18)	15.1(18)	6.0(12)	33(14)
	123	9.02(17)	7.8(16)	15.1(17)	5.0(8)	33(7)
C_{57}: adult mice	120	7.01(12)	6.8(11)	13.1(4)	2.7(2)	13(2)
	121	8.48(7)	8.5(7)		3.4(3)	17(4)

[1] The numerals in parentheses indicate the number of determinations.

DISCUSSION

The relationship between weaning weight and the subsequent requirement for riboflavin emphasizes the importance of careful nutritional standardization of the breeding colony. Minimal variations in the weaning weights of mice may be expected when the breeding stock is maintained on a near-optimal diet. In our efforts to accomplish this we have, however, been struck by the cost of such rations when compared with commercial feeds which all too frequently cannot be called optimal. Our findings with respect to the importance of weaning weights are supported in part by the report of Sherman and Ellis ('39) that the progress of young rats on

riboflavin-deficient diets depended upon the maternal riboflavin intake even though this intake was 3, 7 or 10 times the minimal requirement. Clandinin ('46) found that the early growth rate of chicks was influenced by the maternal riboflavin intake. Although all animals in our breeding colony received the same ration, it may well be that the individual differences in lactation efficiency, which gave rise to different weaning weights of the young, were also reflected in different concentrations of riboflavin in the tissues of the young. Varying tissue vitamin levels at the start of the experiment may be expected to give rise to different growth rates especially on diets containing marginal or submarginal levels of riboflavin.

The reduced food intake of mice on riboflavin-deficient diets has been pointed out by Morris and Robertson ('42–'43) and others. In agreement with our results (fig. 1 E) Mannering and Elvehjem ('44) have reported poor utilization of such deficient diets for growth and maintenance. It is interesting to note that C_{57} strain mice on diet 122 (which just gives maximal growth on this type of ration) ingested in the neighborhood of 500 μg of riboflavin per kg of body weight per day — which is roughly 20 times the level reported for the dog and the monkey. Such a difference in the per kg requirement of riboflavin for 2 species differing widely in body size agrees well with the calculations of Cowgill ('32) for the thiamine requirement.

The loss of body weight of adult mice kept on riboflavin-deficient diets for 9 weeks was found by Morris and Robertson ('42–'43) to be about one-third of the initial weight of C_3H animals. The loss shown by our C_{57} adults was found to be somewhat less. We have observed, however, in unpublished experiments that A strain mice under such conditions lost weight more rapidly than C_{57} animals. It may or may not be significant to mention that both the A and C_3H strains show high incidence of spontaneous mammary tumors whereas the C_{57} strain does not. The lesions we have observed have been adequately reported by Langston, Day and Cosgrove ('33), Lippincott and Morris ('41–'42), and by Jones

et al. ('45), and therefore the description need not be repeated here.

We have observed that A strain mice generally show lower cell counts than do C_{57} mice. One may therefore consider all of the cell counts of the A strain on diets 121, 122 and 123 to be normal. On the other hand the red cell counts of young C_{57} mice were definitely lower on diet 121 than at higher riboflavin levels. The close parallelism between liver riboflavin concentrations and red cell counts does not necessarily suggest a causal relationship. The amount of the vitamin per gm of liver reflects probably the quantity of riboflavin available for functions such as red cell formation. It is a matter of speculation whether the lower tissue riboflavin content of C_{57} mice on diet 121 was due to the fact that these animals showed fairly rapid growth on this diet while the A strain showed only poor growth. This slower growth rate of the A strain may possibly leave more riboflavin available for red cell formation and permit greater tissue saturation at the lower levels of dietary riboflavin.

The strain differences which we have observed here with respect to the riboflavin requirement for growth, red cell formation, and tissue saturation do not necessarily have a direct bearing on the cancer problem. It would be premature to say that the higher vitamin requirements of the A strain determine its cancer susceptibility. It is, however, clearly evident that these observed differences may reflect diverse metabolic patterns, using the term in the widest possible sense, the sum total of which causes one strain to be cancer resistant and the other cancer susceptible.

SUMMARY AND CONCLUSIONS

1. The riboflavin requirement of 2 highly inbred strains of mice has been studied by measuring growth rates at various dietary levels of the vitamin after a 1-week depletion period.

2. Mice of the C_{57} strain showed maximal growth under the conditions of these experiments when the diet contained

0.4 mg of riboflavin per 100 gm, while the A strain required a dietary level of 0.6 mg.

3. At an 0.2 mg level C_{57} mice had lowered red cell counts and muscle and liver riboflavin content, while the A strain showed no such difference.

ACKNOWLEDGMENTS

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COMPARATIVE NUTRITIVE VALUE OF CASEIN AND LACTALBUMIN FOR MAN

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ONE FIGURE

(Received for publication May 12, 1947)

The addition of methionine to a casein hydrolysate, or to a diet of mixed proteins does not increase the efficiency of nitrogen utilization in man (Cox et al., '47; Johnson et al., '47). Since such an addition to intact casein does improve nitrogen utilization in other species (Osborne and Mendel, '15), and since the processing to which a hydrolysate is subjected during the manufacturing process may cause it to differ from the original protein, it is essential to determine the nutritive value of whole casein for man, and to compare it with that of lactalbumin.

EXPERIMENTAL OBSERVATIONS

The casein and lactalbumin employed in the study were commercial products.[1] Analyses for various constituents are summarized in table 1. The casein showed the expected deficiency in cystine but its methionine content and the cystine and methionine content of the lactalbumin, when corrected to a 16% nitrogen basis, agreed closely with the values given by Block and Bolling ('45). Each protein was fed to 20 rats at

[1] Sheffield Farms.

an intake level of 1.2% nitrogen and the difference in growth rate (table 1) clearly indicated that the samples were representative of casein and lactalbumin.

The study was made on 4 adult male volunteers: 3 laboratory workers and 1 clerical worker. All were found normal on the usual clinical examination, and continued their usual duties during the study. One subject contracted an epidemic

TABLE 1

Analytic comparison of casein and lactalbumin.

	CASEIN	LACTALBUMIN
Total nitrogen, % [1]	13.46	12.31
Ash, % [1]	1.77	9.05
Fat, % [1]	1.47	.58
Calcium, % [1]	0.18	0.18
Phosphorus, % [1]	0.80	0.18
Lactose, % [2]	0.4	0.6
Cystine, % [3]	0.2	2.2
Methionine, % [3]	2.8	2.2
Growth of rats, gm/day [4]	1.66	2.74
Gain in wt. per gm N ingested	16.7	21.8

[1] We are indebted to Mr. L. N. BeMiller for these analyses.

[2] We are indebted to Dr. D. L. Morris for this determination.

[3] Analyses by Food Research Laboratories, New York. Cystine was determined by a colorimetric procedure: Block and Bolling ('45); see p. 152. Methionine was determined by a microbiological procedure: Stokes et al. ('45).

[4] Average for 20 rats on each protein fed at level of 1.2% nitrogen for 8 weeks. Diet: Lard, 9; salt mixture, 4; cod liver oil, 2; wheat germ oil, 1; vitamin mixture, 0.2; casein or lactalbumin, 8.92, 9.75; dextrin to make 100.

infection ("influenza") during the last period on lactalbumin, and it was necessary to terminate his part in the study. The subjects were depleted of their protein stores for a period of 12 days, since in an earlier study (Cox et al., '47) a 21-day protein depletion had demonstrated that a steady state of minimal nitrogen excretion was reached in 8 days, and that no good purpose was to be achieved by longer depletion. The protein depletion diet of Mueller, Fickas and Cox ('43) was

employed; the nitrogen intake from the low-nitrogen vegetables and fruits was limited to 0.008 gm nitrogen per kg; and the caloric intake was maintained at 40 cal. per kg body weight. The nitrogen content of coffee and the soft drink used was not considered in the intake and was subtracted from the urinary nitrogen.

A level of supplementation insufficient to produce nitrogen retention was intentionally chosen. It was thought that a continued small loss of nitrogen was preferable to retention for comparative purposes, since we wished to insure a maximal utilization of the ingested protein and maintenance of a reasonably steady condition of nitrogen storage. A further reason was that if positive balances should not be attained, the depletion periods could be used to calculate an average endogenous excretion. The supplementation level was 2% of the calories as the added protein, or 0.033 gm nitrogen per kg. This was 80% of the total protein intake, since 0.008 gm nitrogen per kg came from the basal diet.

In order to rule out the effect of antecedent conditions, each protein was fed alternately for two 4-day periods and each supplemented period was followed by a 4-day depletion period. Thus, there were 4 depletion periods of 4 days each from which the average endogenous urinary and fecal values could be calculated. It was feasible to use all of the depletion periods for this purpose since no storage of nitrogen occurred during supplementation.

Daily determinations of caloric and nitrogen intake (based on analyses of the actual foods employed), and urinary and fecal nitrogen were made. The standard macro-Kjeldahl method was employed for nitrogen, and the fecal samples were prepared for analysis by stirring with concentrated sulfuric acid.

The essential intake and excretion data are given in detail in table 2. The daily balance determinations have been averaged for the 4-day periods. It is readily apparent from the balance column that in none of the periods do the 4 daily balance values differ from the average value more than might

TABLE 2

Urinary and fecal nitrogen excretions and balance during 12 days protein depletion and supplementation with casein and lactalbumin. Values are in grams.

	FOOD N INTAKE, GM SUPPLEMENTAL N, GM Day	SUBJECT A 0.740 3.053 Urine	 Feces	SUBJECT B 0.574 2.366 Urine	 Feces	SUBJECT C 0.711 2.934 Urine	 Feces	SUBJECT D 0.522 2.155 Urine	 Feces	N BALANCE Day	 Period
	1	11.84	2.58	6.78	1.13	7.82	0.21	6.88	1.15	—8.94	
	2	5.82	0.88	4.82	1.13	1.96	1.35	3.88	1.23	—4.60	
	3	5.62	0.78	3.71	0.28	3.94	1.49	3.12	1.49	—4.47	
	4	4.56	2.52	3.65	0.28	3.64	1.36	3.02	0.72	—4.30	
Period	(1) Av.	6.96	1.69	4.74	0.71	4.34	1.10	4.23	1.15		—5.58
	5	4.31	0.66	3.53	0.28	3.65	1.78	2.88	1.16	—3.92	
	6	4.40	0.66	2.86	2.50	3.70	1.78	2.82	1.36	—4.38	
	7	3.41	1.19	2.98	1.47	3.07	1.02	2.67	1.13	—3.60	
	8	3.09	1.70	2.14	0.57	2.59	1.12	2.81	0.91	—3.09	
Period	(2) Av.	3.80	1.05	2.88	1.21	3.25	1.43	2.80	1.14		—3.75
	9	3.07	2.12	2.10	0.57	2.57	1.46	1.92	0.80	—3.02	
	10	3.32	0.96	2.20	0.57	3.11	1.44	2.57	1.02	—3.09	
	11	3.24	0.77	2.35	0.57	2.94	2.49	2.54	1.38	—3.46	
	12	3.86	1.47	2.54	0.31	3.35	0.70	2.82	0.63	—3.32	
Period	(3) Ave.	3.37	1.33	2.30	0.51	2.99	1.52	2.46	0.96		—3.22
Casein	13	3.32	1.28	2.67	1.09	3.40	1.83	2.13	0.86	—0.91	
	14	3.83	0.62	3.22	1.09	3.35	1.33	2.85	0.70	—0.98	
	15	3.42	0.62	2.62	0.37	3.33	0.68	2.98	0.74	—0.43	
	16	3.58	1.55	2.88	0.37	3.37	0.63	3.06	0.98	—0.84	
Period	(4) Av.	3.54	1.02	2.85	0.73	3.36	1.12	2.76	0.82		—0.79
	17	4.05	2.21	3.89	0.37	4.05	1.27	2.84	0.92	—4.26	
	18	3.33	1.12	2.75	0.31	2.54	2.24	2.67	0.77	—3.30	
	19	3.19	1.17	2.96	0.31	2.77	1.20	2.95	1.00	—3.25	
	20	3.25	1.05	2.87	0.36	2.53	0.30	2.19	0.73	—2.74	
Period	(5) Av.	3.46	1.39	3.12	0.34	2.97	1.25	2.66	0.86		—3.39
Lactal-bumin	21	3.19	0.77	2.77	0.14	2.45	2.88	3.34	0.68	—0.79	
	22	3.33	1.05	2.72	0.71	2.64	0.45	2.98	0.62	—0.36	
	23	3.79	1.42	3.00	0.55	2.40	2.95	3.15	1.05	—1.31	
	24	3.42	0.83	2.66	1.07	2.94	0.65	3.22	1.24	—0.74	
Period	(6) Av.	3.43	1.02	2.79	0.62	2.61	1.73	3.17	0.90		—0.80
	25	3.18	1.66	2.46	0.24	2.44	1.89	2.45	0.59	—3.09	
	26	3.24	1.79	2.65	1.47	2.47	0.77	2.37	0.55	—3.19	
	27	2.70	0.72	2.21	0.32	2.38	0.66	2.53	1.01	—2.49	
	28	2.69	0.89	2.09	0.05	2.41	0.77	2.77	0.79	—2.55	
Period	(7) Av.	2.95	1.27	2.35	0.52	2.43	1.02	2.53	0.74		—2.83
Casein	29	2.84	0.98	2.18	0.65	3.20	0.96	2.36	0.88	—0.24	
	30	3.20	1.52	2.81	0.30	3.19	3.04	2.56	0.58	—1.04	
	31	3.49	1.27	2.61	0.25	3.39	0.15	2.84	1.38	—0.58	
	32	3.95	0.91	2.84	1.44	3.25	0.91	2.58	0.67	—0.87	
Period	(8) Av.	3.37	1.17	2.61	0.66	3.26	1.27	2.59	0.88		—0.68
	33	3.84	2.03	2.51	0.20	..[1]	..[1]	2.14	0.50	—3.13	
	34	3.51	1.30	2.78	0.32	..[1]	..[1]	2.06	0.88	—3.00	
	35	3.20	1.16	2.10	0.32	..[1]	..[1]	1.88	0.38	—2.41	
	36	3.56	0.69	2.25	0.21	..[1]	..[1]	2.56	1.02	—2.82	
Period	(9) Av.	3.53	1.29	2.41	0.26	..	..	2.16	0.70		—2.84
Lactal-bumin	37	3.53	1.27	2.46	1.36	..	..	2.58	0.90	—0.90	
	38	3.75	1.57	2.39	0.36	..	..	2.51	0.72	—0.63	
	39	4.12	1.75	2.23	0.57	..	..	2.54	0.82	—0.87	
	40	4.17	0.80	2.34	0.26	..	..	2.64	0.59	—0.46	
Period	(10) Av.	3.89	1.35	2.36	0.64	..	..	2.57	0.76		—0.71

[1] Data omitted because subject contracted "influenza" on thirty-seventh day. Average balance for the 4 days was —2.99 gm.

have been expected. Maximum variation is seen in period 6 (lactalbumin) and period 8 (casein). Even these daily variations, however, are slight. When the data are averaged for each subject, the similarity in performance of the 4 men is even more apparent (table 3). A comparison of the individual balances during the supplemented periods shows that during both casein periods the 4 subjects had very uniform values, but that both lactalbumin periods were characterized by somewhat more variation between individuals. None of the variations were sufficiently large to change the interpretation. The uniformity between individuals under the conditions of this experiment is suggested by the so-called "endogenous" values during the 4 depletion periods. The maximum variations shown by individuals during these periods were only 0.65, 0.78, 1.00 and 0.66 gm (table 3) of nitrogen. This observation is of value in indicating that the subjects were in a steady state of nitrogen metabolism and hence suitable for comparative assay.

The average balances of the 4 subjects are presented in figure 1 by means of the method of graphic presentation suggested by Reifenstein, Albright and Wells ('45). The nitrogen intake is plotted as an area from the zero line toward the bottom of the diagram; the total excretion is indicated by the height of the column above the intake level, and the urinary and fecal values are indicated by the waved line. In this arrangement the projections of the columns above the zero line represent negative balances. A positive balance is shown by that amount by which the total height of the column does not reach the zero line. Since all balances in this study were negative, the differences in the height of the columns above the zero line are of major interest. The most pertinent portion of the columns have been darkened merely for emphasis.

One can see by inspection that the balances with casein and lactalbumin are identical for all practical purposes. Both fecal and urinary excretions show exactly the same pattern of changes with both proteins. There is 1 exception, namely, the high urinary nitrogen during the first depletion period follow-

ing casein supplementation. This may be partially explained on the basis of a transient infection shown by 1 subject who experienced loose stools, otorrhea and rhinitis on this day. One other subject on this day reported that he was developing a cold. This explanation is probably valid since the depletion period following the administration of casein for a second time gave average excretion values consistent with those following other supplementation periods.

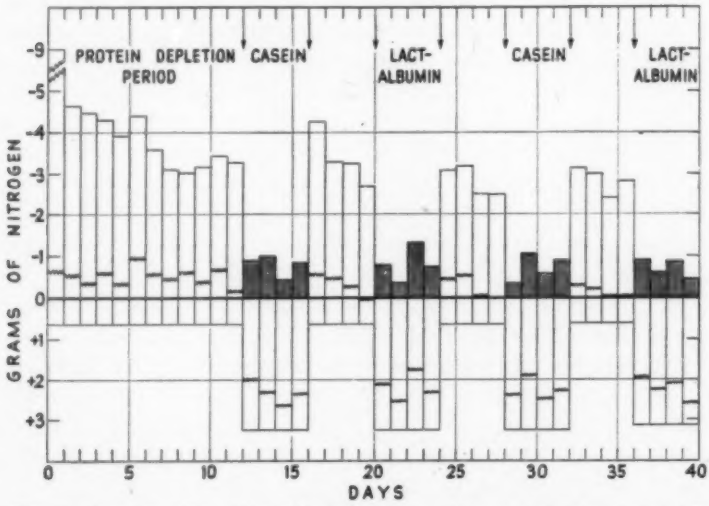

Fig. 1 Average daily nitrogen intake, nitrogen balance and urinary and fecal nitrogen excretion of 4 healthy men during protein depletion and subsequent supplementation with casein and with lactalbumin. Protein was added to the basal diet (0.008 gm nitrogen per kg) to supply 0.033 gm N per kg body weight. See text for construction of the figure.

The figure clearly indicates also that the fecal excretion during the depletion periods was always greater than the nitrogen intake, strongly suggesting that much or all of the nitrogen of the basal diet was not available for utilization.

Table 3 gives the calculated biological values. We have employed the method of Mitchell ('24), and for the individual calculations used the average endogenous urinary and fecal

nitrogen of each subject for the 4 depletion periods. The values show good agreement. The maximum variation between individuals on a single assay (which is also the greatest variation in the whole series) was observed in the first lactalbumin period as that between 100 and 72.3. This variation approximates that reported by Mitchell ('24, table 12) when the assay method was first described. The average biological values for the 2 casein periods were 89.0 and 94.7, and for the 2 lactalbumin periods 90.2 and 93.3. With the variables known to exist in this type of assay, these values must be regarded as practically identical.

TABLE 3

Nitrogen balances during depletion and supplementation, and biological values.

SUBJECT	A	B	C	D	AVERAGE B. V.[1]
	All nitrogen values are in grams per day				
Depletion (9–12 day)	—3.92	—2.26	—3.81	—2.90	
Casein	—0.76	—0.64	—0.83	—0.93	
B. V.	(94.5)	(88.5)	(84.6)	(88.4)	89.0
Depletion (17–20 day)	—4.13	—2.88	—3.52	—3.00	
Lactalbumin	—0.65	—0.47	—0.69	—1.40	
B. V.	(97.4)	(91.2)	(100)	(72.3)	90.2
Depletion (25–28 day)	—3.48	—2.29	—2.81	—2.75	
Casein	—0.74	—0.32	—0.87	—0.79	
B. V.	(98.9)	(97.8)	(87.3)	(94.7)	94.7
Depletion (33–36 day)	—4.09	—2.10	.....	—2.34	
Lactalbumin	—1.44	—0.05	.....	—0.66	
B. V.	(84.4)	(100)	.....	(95.5)	93.3
Av. depletion	—3.91	—2.38	—3.38	—2.75	

[1] Biological value.

DISCUSSION

Early observations (Osborne and Mendel, '15) indicated that casein and lactalbumin were nutritively different when measured by the growth of rats, and this finding was subsequently extended to include dogs (Lewis, '17), mice (Bauer and Berg, '43) and chicks (Almquist, '42). It was only natural to interpret these animal findings in terms of human nutrition,

since there is a preponderance of casein in cow's milk and the lactalbumin content of mother's milk is higher than its casein content. Since clinical experience indicated that better results attended the feeding of infants with mother's milk, it was logical to attribute part of the superiority to a higher content of lactalbumin (Holt, '16).

The nutritive value of the 2 milk proteins in the feeding of infants was first put to experimental test by Edelstein and Langstein ('19). These investigators reported nitrogen balance studies on 4 infants who received varying levels of either casein or lactalbumin for various periods. Their one best experiment was with an 11-week-old child who was given a protein-free diet (cane sugar, carrots, starch, butter and salt solution) for 12 days, followed by an 18-day period on casein, a 6-day protein-free period, and a final 13-day lactalbumin period. We have recalculated their data to obtain biological values comparable with those reported here: for casein the value was 85.0; for lactalbumin, 87.4. During the 18-day casein period, the infant gained 500 gm in weight; during the lactalbumin period, 190 gm. Two additional infants, observed only for single 3-day periods, had recalculated biological values for casein of 77.4 and 65.3, and for lactalbumin of 98.2 and 67.0, respectively. Observations on the fourth infant were so interrupted by technical difficulties as to be uninterpretable. The authors concluded that lactalbumin was superior to casein for infant nutrition. Our study of their data in which we made no selection of values, does not support such an interpretation. With consideration of the difficulties of working with infants and of the variation in biological values even in adults under very uniform conditions, their data do not demonstrate to us any significant difference in the 2 proteins in infants.

It was not until Harrison ('36) showed that adequate nitrogen retention could be attained by feeding cow's milk proteins to infants at a low intake level, and Gordon, Levine, Wheatley and Marples ('37) showed that similar nitrogen retention in premature infants could be attained by feeding equal levels of nitrogen as cow's milk and as mother's milk that the earlier

concepts of the different nutritive qualities of the 2 proteins for man became less tenable.

It has been repeatedly demonstrated that the nutritive value of casein for rats or dogs can be made equal to that of lactalbumin by the addition of a small amount of cystine or methionine. Using a casein hydrolysate supplemented with methionine, Cox and associates ('47) showed that no effective improvement in nitrogen retention resulted from the added methionine when the hydrolysate alone or supplemented with this amino acid was administered to 4 groups of persons of varying protein need: (1) infants; (2) adults on a maintenance nitrogen level; (3) protein-depleted adults allowed less nitrogen than that necessary to attain nitrogen equilibrium; and (4) adults nourished solely by intravenous feedings. While this evidence strongly suggested that intact casein might be the nutritional equivalent of intact lactalbumin in man, it could not substitute for an actual experimental comparison of the 2 proteins.

The present experiment in which the 2 proteins have been compared at low intake levels in healthy but slightly protein-depleted adults, leaves no doubt that the 2 substances are of equal value for adult maintenance. It is possible that a comparison at higher intake levels would disclose some difference, but our earlier findings (Cox et al., '47) with a methionine supplemented casein hydrolysate at different intake levels would certainly indicate that this is unlikely. A new comparison of the 2 proteins should be made in infants, since the requirements of the growing child may be different from those of the adult. However, in view of the work cited above (Edelstein and Langstein, '19; Harrison, '36; Gordon et al., '37; Cox et al., '47) it is unlikely that any difference between the 2 proteins will be found even in infants.

SUMMARY AND CONCLUSION

Casein and lactalbumin have been fed at an intake level of 2% of the calories to 4 well adults following a 12-day protein depletion. Two 4-day periods of supplementation with each protein were alternated with 4-day depletion periods. Nitro-

gen balances and calculated biological values indicated that casein and lactalbumin are equally effective in maintaining nitrogen balance in adult man.

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THE GROWTH-PROMOTING ACTION OF CELLULOSE IN PURIFIED DIETS FOR CHICKS [1]

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Studies on "crude fiber" in rations of small laboratory animals have been reported previously, but there exists in the literature much controversy as to its need and utilization by poultry. It is generally understood that excessive levels of "crude fiber" in poultry rations reduce feed efficiency, growth, and egg production. On the other hand, the presence of "crude fiber" appears to be beneficial under certain conditions. For example, Sheehy ('39) and Bearse, Miller and McCleary ('40) observed that cannibalism is prevented by the introduction of extra "crude fiber" in the ration. However, such studies have been made with practical-type poultry rations, and the fiber supplement may have contained effective nutrients other than fiber.

In the study presented here, a purified source of cellulose was added to a "synthetic" ration nutritionally complete in all the known nutrients and free of fiber. The results show that the addition of cellulose to such a ration improved the growth rate and was otherwise beneficial.

Woolley and Sprince ('45) showed that 20% of cellulose supplied as Cellu-flour or powdered cellophane in purified guinea pig rations was active as a growth stimulant. No other report on the growth promoting-action of cellulose in purified

[1] Scientific paper no. A165. Contribution no. 2060 of the Maryland Agricultural Experiment Station (Department of Poultry Husbandry).

rations for small laboratory animals (non-ruminants) is available. Workers in various laboratories have included cellulose materials in purified poultry rations but have not made any reports on its growth-promoting effect.

EXPERIMENTAL PROCEDURE AND RESULTS

Day-old New Hampshire chicks were reared in electrically heated batteries with raised screen floors. Feed and water were supplied *ad libitum* throughout the 4-week experimental period.

TABLE 1

Composition of basal ration 113.

Main ingredients	%	*Supplements*	*mg/100 gm*
Glucose ("Cerelose")	61.4	Thiamine HCl	0.4
Casein (erude)	18.0	Riboflavin	0.8
Gelatin	10.0	Ca pantothenate	2.0
Soybean oil	4.0	Choline Cl	200.0
Salts 1 M (Briggs, '46)	6.0	Nicotinic acid	5.0
l(—)Cystine	0.3	Pyridoxine HCl	0.6
		Biotin	0.02
		Folic acid	0.2
		i-Inositol	100.0
		Para-aminobenzoic acid	0.2
		Alpha-tocopherol	0.5
		2-Methyl-1, 4-naphthoquinone	0.1
	Vitamin A in I.U. 1200 [1]		
	Vitamin D_3 in A.O.A.C. units 170 [1]		

[1] Vitamins A and D_3 are fed by dropper weekly.

The basal ration (113) given in table 1 was used in the experiments and contains all nutrients known to be required by the chick. All supplements with cellulose [2] were made at the expense of glucose. Feed consumption records were taken in order to obtain feed efficiency data, and the chicks were weighed individually each week.

[2] "Ruffex," a roughage material distributed by the Fisher Scientific Co., Pittsburgh, Pa., was used. It is processed from rice hulls and contains 70% alpha cellulose, the balance being simple and hydrocelluloses. It is reported to contain neither proteins, fats nor vitamins and gives an ash value of less than 1%.

The results of 2 series, summarized in table 2, show that the addition of from 5 to 15% of cellulose in the ration resulted in increased growth and apparently greater feed efficiency values. The greatest response in growth was at the 5% level of cellulose. The 10% and the 15% levels of the cellulose also gave better growth and feed efficiencies when compared with the basal ration. Increasing additions of the supplement through 50% resulted in decreased growth, decreased feed efficiency values, and smaller statures when compared with chicks in group 1. More recent feeding trials (not shown in the table) have given similar growth responses with cellulose.

TABLE 2

Summary of the effect of cellulose supplements in purified chick rations.

GROUP	SUPPLEMENT TO BASAL DIET 113	NUMBER CHICKS STARTED	NUMBER DEAD AT 4 WEEKS	AVERAGE WEIGHT AT 4 WEEKS	FEED EFFICIENCY [1]
				gm	
1	None	16	2	308	0.491
2	5% Cellulose	16	0	354	0.587
3	10% Cellulose	16	0	340	0.560
4	15% Cellulose	16	0	340	0.564
5	20% Cellulose	16	0	288	0.434
6	30% Cellulose	12	0	268	0.311
7	40% Cellulose	12	0	200	0.312
8	50% Cellulose	12	1	140	0.215

[1] $\frac{\text{Total gain in weight}}{\text{Total feed intake}}$.

No difference in feathering and no feather picking or cannibalism were observed in any of the experimental groups. It is interesting to note that as high as 50% of cellulose was consumed with practically no mortality, although growth was poor. As much as 15% of cellulose in the ration was tolerated without any ill effects. Statistical treatment given in table 3 showed that the 5%, 10% and 15% supplemental levels gave significantly greater growth responses compared with group 1.

The droppings of the chicks on all levels of cellulose supplements were of a drier, firmer consistency than those of the controls. Feed consumption values and chemical analysis of

the droppings for a 1-week period gave digestibilities of 53.9%, 27.9%, and 34.3% for the "crude fiber" fed at the 5%, 10% and 15% levels of cellulose, respectively. Analysis of the cellulose for "crude fiber" gave a value of 75.38%.[3]

TABLE 3

Analysis of variance.

SOURCE OF VARIATION	D.F.	VARIANCE
Treatment	3	5502.33 [1]
Error	58	1796.59

[1] Significant to 0.05 level.

DISCUSSION

The exact reason for the increased growth obtained by feeding cellulose is as yet unknown, but it is believed to be due to the presence of cellulose itself rather than to a possible contaminant (unpublished data). It is possible that hydrolysis of the alpha cellulose and of the simple and hydro-celluloses in the intestinal tract gives rise to small amounts of growth stimulatory product(s) other than glucose or other than products that may be derived from glucose. Enzymes of microbiological or intestinal origin, or both, may be active in such hydrolyses. It is known that certain microorganisms produce cellulose-splitting enzymes. In addition, Baker ('42) and Hungate ('44, '46) have shown that various microorganisms possess the ability to metabolize cellulose. One or more of the decomposition products derived from cellulose metabolism may act as growth factors.

It is also possible that the mere physical presence of the cellulose may be beneficial in some manner. For instance, its presence in restricted amounts could aid in the intestinal absorption of the nutrients. However, the feed efficiency values for the 5%, 10% and 15% levels of cellulose given in table 2 show that cellulose is probably not functioning simply as a bulk factor.

[3] We wish to thank Robert G. Fuerst for these analyses.

The retarded growth and lowered feed efficiency values with the feeding of the 20% through 50% levels of cellulose were probably caused by a decrease in the availability of metabolizable simple carbohydrates, since the supplements were fed at the expense of glucose.

This work shows the importance of including some source of cellulose (or perhaps its decomposition products) in purified laboratory rations in order to obtain maximum growth and feed efficiency. In addition, the beneficial effect of limited amounts of fiber in practical rations may be more easily studied and better understood in view of this finding.

SUMMARY

The addition of 5%, 10% and 15% of cellulose to a purified chick diet free of fiber but complete in all known nutrients resulted in a significant increase in growth. Additions of cellulose at levels ranging from 20% through 50% resulted in retarded growth but there was practically no mortality in these groups under the experimental conditions described. Possible reasons for the growth-promoting effect of cellulose are discussed.

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A STUDY OF THE FASTING-HOUR EXCRETION OF THIAMINE IN THE URINE OF NORMAL SUBJECTS [1]

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ONE FIGURE

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A number of excretion tests have been used as a measure of nutritional status with respect to thiamine. All are based upon the premise that the amount of thiamine available for excretion by the kidney depends more or less upon the adequacy of the supply in the body (Melnick, '42). Of all the procedures employed the fasting-hour excretion test, first recommended by Holt and Najjar ('42), appears to be the most feasible for nutritional surveys.

Melnick and Field ('42) reported good correlation between 24-hour output and fasting excretion of thiamine in 37 subjects. The fasting specimens in this study, however, represented a 4-hour period. Oldham and her coworkers ('44) in their study of the thiamine requirement with 2 young subjects of pre-school age concluded that the 1-hour fasting excretion test reflected the nutritional status with respect to thiamine. To our knowledge no one has reported any extensive comparison of the 24-hour urinary excretion of this vitamin with that in a 1-hour specimen collected immediately after the completion of the 24-hour period and following an overnight fast. Najjar and Holt ('43) apparently accumulated such data but we have not seen these published. Since the daily

[1] Aided by grants from the Nutrition Foundation, Inc., and from the University Center in Georgia.

urinary output of thiamine has been used widely in assessing thiamine nutritional status (Allen, '43; Elsom et al., '42; Jolliffe et al., '39; Melnick and Field, '42), it seemed to us worthwhile to compare this value with that of a fasting 1-hour specimen collected in the same metabolic period by normal individuals. This paper presents such values obtained from 63 young adults. In an attempt to more properly evaluate the fasting-hour excretion test with the view of its application to survey work, a 4-hour clearance test following an oral test dose of 1 mg of thiamine hydrochloride was also made on 20 of the subjects.

PROCEDURE AND METHODS

The subjects were 59 young men and 4 young women between the ages of 19 and 28. They were all university students, and none had apparent signs of any vitamin deficiency. No attempt was made to control the diet since the 24-hour specimen and the subsequent fasting-hour specimen from each individual were both collected during the same metabolic period. The 24-hour urine collections were made in the conventional manner, beginning after the voiding of the morning specimen of 1 day and ending with the morning urine of the following day. The fasting-hour urines were collected essentially according to the procedure recommended by Holt and Najjar ('42). The subjects were instructed to abstain from food or beverages except water beginning with the fourteenth hour of the 24-hour period. Upon completion of the 24-hour collection they were asked to note the time and drink 1 glass of water. The fast was continued for another hour at the end of which the fasting specimen was collected. This urine sample thus represented the hourly output at the end of a fast of 11–13 hours' duration. Where the fasting specimen represented more or less than 60 minutes, the time of voiding was recorded and the hourly fasting urinary output was calculated.

In the clearance tests, the subjects came to the laboratory before the completion of the fasting hour, that is, the twenty-

fifth hour of the total period. When this hour was completed and the fasting specimen had been collected, the subjects were given 1 mg of thiamine hydrochloride in solution by mouth. All the urine voided during the next 4 hours was collected and analyzed for urinary thiamine. During this clearance period only water and black coffee were allowed.

The specimens were preserved with glacial acetic acid (2–3 ml per 100 ml) and stored in dark bottles in the refrigerator until analysis for thiamine was made. The analyses were all completed within 5 weeks of the day of collection. We have been able to verify the statement of Egaña and Meiklejohn ('41) that the thiamine content of urine thus preserved remains unaltered for more than 6 weeks.

The thiamine content of the specimens collected by the first 43 subjects was determined by the Merck and Company, Inc., adaptation ('41) of the Hennessy and Cerecedo thiochrome method (Hennessy, '41). Calculations, however, were made according to the formula of Najjar and Ketron ('44) which is believed to correct for the reduction of fluorescence caused by oxidation of F_2(N^1-methylnicotinamide). The specimens of the last 20 subjects were analyzed by a fluorometric adaptation (Papageorge and Lamar, '47) of the Urban and Goldman ('44) thiochrome method which employs benzenesulfonyl chloride for the destruction of thiamine in the blank determination.

RESULTS

Figure 1 presents the comparison of the 24-hour with that of the fasting 1-hour excretion of urinary thiamine in the 63 subjects studied. The scatter diagram shows that there is a definite correlation between the 2 values, particularly at lower levels of excretion. Assuming a linear relationship between the 1-hour fasting excretion of thiamine and the 24-hour output, the coefficient of correlation [2] is 0.70. This

[2] Coefficient of correlation was calculated according to the equation

$$r_{xy} = \frac{N\Sigma XY - (\Sigma X)(\Sigma Y)}{\sqrt{[N\Sigma X^2 - (\Sigma X)^2] \quad [N\Sigma Y^2 - (\Sigma Y)^2]}}$$

Guilford, J. P. ('42) Fundamental Statistics in Psychology and Education, p. 204. McGraw-Hill Co., New York.

indicates good correlation. The 11 points representing the lowest 24-hour and fasting 1-hour excretion values observed all fall within the 2 regression lines.[3]

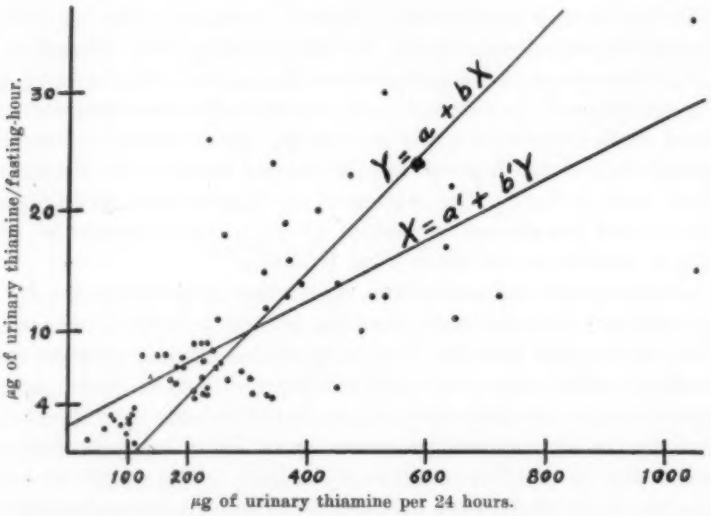

Fig. 1 Correlation of 24-hour with fasting-hour excretion of thiamine in the urine of 63 subjects. Coefficient of correlation is 0.70. Intersection of regression lines indicates mean values.

Table 1 gives the minimum, maximum and mean values of the whole series. From the mean values as well as from examination of figure 1 it is seen that the 24-hour excretion is usually more than 24 times the value of the fasting 1-hour excretion. This is not surprising since the intake of thiamine during the period of collection would be expected to influence the 24-hour output to a greater degree than it would affect the excretion after 12 hours of fasting. However, in 14 of the

[3] Regression lines are lines of trend along which lie the best predictions of 1 value from the other. They intersect at the point representing the mean values of the 2 variables. Since the slopes of the lines depend upon the coefficient of correlation, the higher this coefficient, the closer together will a pair of regression lines tend to lie. See Guilford, pp. 211 ff.

subjects the 24-hour value was less than 24 times that of the subsequent fasting-hour excretion of thiamine. All of these subjects excreted more than 120 μg of thiamine per day and thus over 5 μg per fasting-hour.

Table 2 presents the results found on the last 20 subjects on whom a clearance test was also made. Urine volumes are included in the table since they may have some bearing on the explanation of certain of the discrepancies observed between the 3 tests of thiamine status. For some reason the fasting-

TABLE 1

Minimum, maximum and mean values of urinary thiamine excreted per day and per subsequent fasting-hour by 63 subjects.

	THIAMINE EXCRETION	
	μg/24 hrs.	μg/fasting-hour
Minimum 24-hr. value	33	1.1
Minimum fasting-hour value	109	0.7
Maximum 24-hr. value	1050	15.0
Maximum fasting-hour value	1044	36.0
Means of 63 values	309	10.1

hour volumes of these last 20 subjects were on the whole much smaller than those of the first 43 individuals studied. The correlation coefficients for the thiamine excretion values in table 2 are as follows: 0.80 for correlation of fasting-hour with 24-hour output; 0.64 for correlation of percentage of test dose excreted in 4 hours with amount of thiamine excreted during the fasting-hour; 0.77 for correlation of percentage of test dose return with 24-hour output of the vitamin.

DISCUSSION

Assuming that the 24-hour excretion of thiamine is a fair index of nutritional status with respect to this vitamin, our results indicate that the fasting-hour excretion test can be employed in assessing thiamine status where a large number of people are to be tested. This conclusion agrees with the findings of Melnick and Field ('42) on 4-hour specimens and with the statement of Oldham et al. ('44) based upon the

correlation of fasting 1-hour excretion with the 4-hour return of a test dose and with thiamine intake in their 2 small subjects.

To be sure, the measure of the urinary output of thiamine as an index of nutritional status is definitely limited (Elsom et al., '42). Rate of absorption and rate of excretion by the kidney are undoubtedly involved as well as level of intake and rate of utilization. While, on the whole, our data confirm the observations of others (Elsom et al., '42; Youmans et al., '40) that the amount of thiamine excreted is independent of urinary volume, there are indications that this may not hold true outside of ordinary limits. One may well ask if in instances where the fasting-hour volume is excessively small, such as

TABLE 2

Urinary excretion of thiamine per 24 hours and per subsequent fasting-hour with 4-hour urinary return of 1 mg oral test dose of thiamine in 20 subjects.

SUBJECT NO.	24 HR. URINE		FASTING-HOUR URINE		CLEARANCE URINE	
	Vol.	Thiamine	Vol.	Thiamine	Vol.	Return of 1 mg dose
	ml	*μg*	*ml*	*μg*	*ml*	%
44	2000	178	81	5.7	620	10.4
45	2000	192	91.5	7.8	590	9.7
46	2000	243	31	8.4	288	6.0
47	2000	450	35	5.2	500	12.4
48	2000	469	99	23.0	140	11.4
49	2200	581	151	24.0	354	15.0
50	1820	530	51.5	13.0	178	11.1
51	2000	87	31.5	2.2	130	5.1
52	2000	325	86	15.0	760	14.1
53	1500	109	14	0.7	123	3.4
54	1500	307	43.5	4.8	334	9.7
55	1000	231	37.5	5.1	187	7.1
56	790	33	35	1.1	815	7.1
57	2000	338	22.5	4.6	420	14.5
58	1000	77	57	2.9	410	4.4
59	1000	102	17	2.6	128	3.3
60	1500	106	49.5	3.0	184	3.2
61	1500	229	58	4.8	415	9.5
62	2200	224	76	4.8	149	6.8
63	1000	98	17	1.5	210	9.3
Mean values		245		7.01		8.6

in subjects 53, 59 and 63, there may not have been a delay in excretion of thiamine; or, if in cases like that of subject 56 the relatively high return of the test dose may not have been due to diuresis since the clearance specimen of this individual was greater in volume than her total 24-hour collection. The completeness with which the bladder is emptied may also be a factor in determining the accuracy of results representing short periods of urinary excretion as in the case of subject 47 whose fasting-hour value was lower than one would expect on the basis of his daily output and his 4-hour test dose return.

The results of the clearance test do not throw much light on the question of the comparative value of the fasting-hour excretion and the 24-hour output as measures of thiamine status. While statistically the clearance test showed better correlation with the 24-hour than with the fasting-hour excretion, our data are open to criticism in view of the small amount of test dose (1 mg) administered orally. There is also the question of rate of excretion of vitamin in instances of extraordinarily low or extremely high urinary volumes. Gifft and Hauck ('46) failed to obtain agreement in their comparison of 4 methods for studying urinary excretion of thiamine, 2 of which were essentially clearance procedures. It would seem, however, that the fasting-hour output should be less liable to fluctuations caused by sudden changes in intake than the 24-hour excretion (Holt, '43).

In attempting to arrive at a fasting-hour level of thiamine excretion which should represent the division between adequate and inadequate values, we may use established daily output as a basis. It is generally agreed that on adequate intakes the daily excretion of thiamine in urine exceeds 100 μg and is usually around 200 μg or more (Allen, '43; Gifft and Hauck, '46; Jolliffe et al., '39; Mason and Williams, '42; Melnick and Field, '42). According to the revised daily allowances recommended by the Food and Nutrition Board of the National Research Council (Jeans, '46), the intake of our subjects, if adequate, should have been at least 1.0 to 1.5 mg

of thiamine per day. Such intakes should result in 200 to 300 μg daily excretion in the urine on the 20% basis of Melnick, Field and Robinson ('39). If we use the regression line $Y = a + bX$ in figure 1 to get values of Y from X, the predicted fasting-hour output for the 200–300 μg 24-hour excretion is 4.5 to 9. μg of thiamine. The mean values for the 63 subjects are not far from these figures: 309 μg per 24 hours and 10.1 μg per fasting-hour.

Eleven of the subjects excreted less than 4 μg of thiamine per fasting-hour. Seven of these had 24-hour excretions less than 100 μg of the vitamin and the other 5 showed between 100 and 110 μg in their 24-hour specimens. Clearance tests were made on 7 of the 11 subjects with low daily and fasting-hour thiamine excretion. Five of the 7 gave less than a 6% return of the test dose. The other 2 were subjects 56 and 63 in whom the higher test dose return may have been due to diuresis or to delayed thiamine excretion, respectively, because of their unusual urine volumes. No subject excreting over 4 μg of thiamine during the fasting hour gave less than a 6% return of the oral test dose of 1 mg.

These data suggest that 4 μg may be the critical level of fasting 1-hour excretion of thiamine. This value agrees with the findings of Melnick and Field ('42) who place the minimal normal level of 4-hour fasting excretion at 15 μg. Oldham and her coworkers ('44) observed higher fasting 1-hour excretions in their 2 young subjects when the children were on an adequate allowance of thiamine. The obvious limitations of the fasting-hour excretion test as a measure of thiamine nutritional level should be borne in mind. It can be used only as an approximate index for clinical purposes and should be repeated in conjunction with a standard clearance procedure in cases of suspected thiamine deficiency. However, it offers itself as a useful tool in estimating the general trend of thiamine level in survey studies.

SUMMARY

1. The 24-hour urinary output of thiamine of 63 normal young adults was compared with that in a "fasting-hour"

specimen, collected in the hour following completion of the 24-hour period and after an over-night fast. The 2 values show good over-all correlation with a coefficient of 0.70.

2. The percentage of a 1-mg oral test dose of thiamine returned in 4 hours in the fasting state was also determined in 20 of the 63 subjects. The correlation between the test dose return and the 24-hour excretion was better than that between the test dose return and the fasting-hour output. However, the procedure followed in the clearance test is open to criticism.

3. The fasting-hour excretion test offers itself as a convenient method of estimating thiamine nutritional status in survey work where a large number of individuals are to be tested.

4. The critical level of fasting-hour excretion of thiamine appears to be 4 μg. Values below this suggest a likelihood of inadequate thiamine intake.

ACKNOWLEDGMENTS

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THE AVAILABILITY OF VITAMINS FROM YEASTS

III. THE AVAILABILITY TO HUMAN SUBJECTS OF RIBOFLAVIN FROM FRESH AND DRIED BAKERS' YEASTS VARYING IN VIABILITY [1]

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ONE FIGURE

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In continuation of earlier investigations (Parsons and Collord, '42) in which riboflavin of yeast was found to be less available for absorption by human subjects from the fresh living cells than from non-living cells, a wider variety of yeast types have been tested. These included samples in which the riboflavin had been acquired by the yeast gradually during growth and hence was presumably inside the cell (Massock, '43) in contrast to other samples in which the greater part of the riboflavin had been added in fortification; and dried yeasts, either with dead cells only, or with a suitable degree of viability for leavening purposes. These samples gave new opportunity for observation on possible correlation of the condition of the yeast cell with the availability of its riboflavin.

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EXPERIMENTAL

Two general plans for metabolic studies were employed: 1 used previously in this laboratory in which periods of 3 to 5 days were utilized for the uniform feeding of the basal diet alone or supplemented by a given riboflavin dose; and the other devised by Melnick et al. ('45), wherein a single test-day was preceded and followed by periods of the basal diet alone. The first technic was used in the experiment which is recorded in figure 1; the second in those recorded in tables 2, 3 and 4. Riboflavin excretions for a total of approximately 360 subject-days were assayed.

The test-doses included various types of yeast as well as a positive control dose of pure riboflavin. The identities and descriptions of these yeasts are to be found in table 1 of the following paper (Kingsley and Parsons, '47). The yeasts of the series I-A, I-B, I-C and II-C were produced for the experiment in the factory and the first 3 were from 1 lot, hence yeast strain and conditions of production were ruled out as variables. Viability and moisture content were intentionally varied in the different samples: yeast I-A was a fresh raw yeast, I-B and V-B yeasts dried with little loss of viability, and I-C and II-C with entire loss of viability.

The subjects were university students, men and women, in a satisfactory nutritional state. The basal diet is listed in table 1. Uniformity of intake was sought by the use of unit lots of food: flour,[3] and canned peas,[4] tomato juice,[4] pineapple [5] and applesauce. Supplies of meat loaf, orange-cranberry relish and ice cream [6] were prepared and frozen at the outset of the study to be used throughout.

[3] Patent white flour without fortification was obtained direct from the Pillsbury Mills, Inc., at Minneapolis. Bread was baked in the laboratory 2 to 3 times per week.

[4] Uniform packs of peas and tomato juice were furnished through the courtesy of the Research Department of Libby, McNeill and Libby Company.

[5] Thanks are due to the Pineapple Research Institute of Hawaii for the gift of the specially packed crushed pineapple and pineapple juice.

[6] Prepared from a special formula under the direction of Mr. L. C. Thomsen, Associate Professor of Dairy Industry, University of Wisconsin.

The 24-hour urinary excretions were collected in brown bottles containing acetic acid and chloroform, and a representative sample was preserved with the addition of toluol. Fecal collections were marked by the ingestion of carmine at the beginning and end of each basal and yeast period. The fecal material was preserved in acid-alcohol and assays were made

TABLE 1

Composition of basal ration.

Breakfast	gm
Tomato juice	200
Egg, 1 medium	
Bread, ½ loaf	
Butter	10
Milk	200
Dinner	
Pineapple juice or crushed pineapple	100
Meat loaf	100
(Beef and veal 68)	
(Pork 7)	
(Egg 10)	
(Oatmeal or soygrits 15)	
Cranberry relish	100
(Cranberries 50)	
(Oranges 33)	
(Sugar 17)	
Peas	100
Ice cream	100
Bread, ½ loaf	
Butter	10
Milk	200

Supper	gm
Apple sauce	100
Milk	200
Peanut butter	25
Butter	10
Bread, ½ loaf	

Ingredients 1 loaf bread (in gm):	
Unfortified white flour	113
Yeast F	3
Lard	4
Sugar	4
Salt	2
Milk (plus water as needed)	39

Analyses conducted on aliquots of the composite diet	
Protein (N × 6.25)	approx. 72 gm
Thiamine	1.2 to 1.5 mg
Riboflavin	3.7 to 4.2 mg

from a thoroughly mixed representative sample Collection of fecal eliminations was of necessity omitted during the experiments employing the 1-day test-dose period.

Riboflavin was determined by the fluorometric method of Conner and Straub ('41). Duplicate determinations were made on separate days for all samples. Food aliquots representing 25% or 100% of the weighed daily food intake were homogenized in a Waring Blendor and assayed for riboflavin.

RESULTS AND DISCUSSION

Figure 1 shows that, in confirmation of previous results with other fresh yeast samples (Parsons and Collord, '42), the riboflavin which was unavailable from the live, raw, fresh yeast I-A was rendered available by treatment of the yeast with heat prior to ingestion. It is evident that the tenacity of the live yeast cell in withholding the 825 μg of riboflavin in 45 gm of yeast I-A was abolished by the heat-treatment of the yeast and hence the riboflavin was released for absorption.

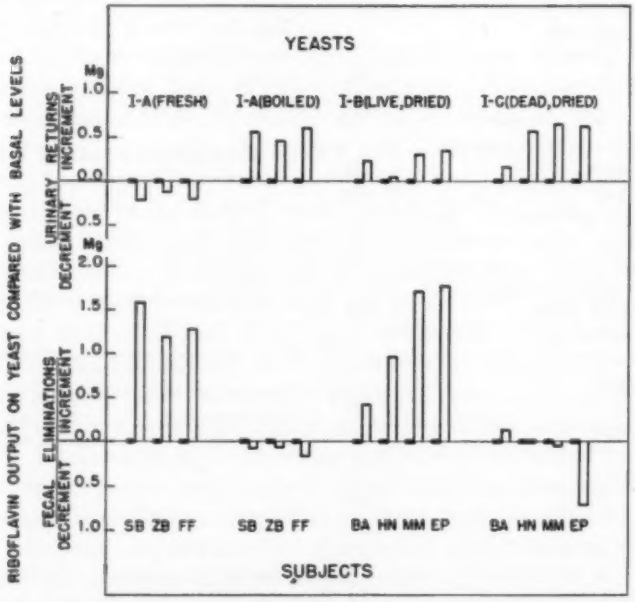

Fig. 1 This shows the general reciprocal relationship between the urinary excretion of riboflavin (top of chart) and fecal elimination (bottom of chart) on a basal diet with supplements of fresh and dried yeasts in viable and non-viable forms derived from 1 factory-lot. The viable yeast samples, both fresh and dried, led, in general, to higher fecal outputs and lower urinary excretions than did the non-viable samples. The open bars represent the average increments or decrements in riboflavin output for 5-day periods as compared with the values on the basal diet, which are used as the base line and are represented in figure 1 by heavy bars.

The riboflavin of dried yeast I-C, which contained only dead cells, appeared to be completely available for absorption for 3 of the 4 subjects ingesting the yeast (fig. 1). On the other hand, the riboflavin of dried yeast I-B containing live cells was only partially available as judged by the comparatively low urinary riboflavin during this yeast period. Hence it seems that the relative availability of riboflavin in dried yeasts is not essentially due to the drying as such but is more closely correlated with the effect of the process on the viability of the yeast cell.

This general hypothesis was given a certain degree of confirmation by the results on fecal riboflavin content. During the feeding of live yeasts I-A and I-B there was a striking increase in fecal riboflavin although the urinary output had been shown to be relatively low. In contrast, during the feeding of dead yeasts I-A (after boiling) and I-C the fecal riboflavin values did not increase over the basal levels, but the urinary output for 6 of the 7 subjects was exceptionally high. This reciprocal relationship between urinary and fecal elimination appears to rest on 2 factors: The high fecal values for riboflavin are probably derived most largely from that portion of riboflavin held tenaciously by the live yeast cell and eliminated unabsorbed from the digestive tract. However, inasmuch as calculations show that the increment cannot be attributed solely to this source, it seems evident that some fecal riboflavin originated from intestinal synthesis. Nevertheless, the very fact that such synthesis was stimulated by the eating of live yeast but not by the same yeast with dead cells only, is evidence in itself that live yeast in the digestive tract tends to escape early digestion and absorption and hence, far down in the digestive tract, may furnish a favorable nutritive medium for the multiplication of bacteria. Although the high fecal riboflavin was indicative of synthesis during the ingestion of yeast I-A, it is significant that this riboflavin was not absorbed as it did not increase the urinary output above the level due to the basal diet alone. Dead yeast cells did not seem to contribute significantly to the

nutritive medium in the lower bowel, nor did they carry their content of riboflavin out of the tract with the feces.

The human bioassay technic of Melnick et al. ('45) with the simplified dosage routine was also used to re-test the availability of these yeasts. Using this method, the percentage excretion of a pure riboflavin control test-dose by both male and female subjects was found to be in good agreement with similar studies reported by these authors.

TABLE 2

Availability to human subjects of riboflavin of raw, fresh yeast I–A.

SUBJECT	CONTROL PERIOD: 3.0 MG PURE RIBOFLAVIN IN SOLUTION			TEST PERIOD: 2.6 MG RIBOFLAVIN IN 150 GM RAW, FRESH YEAST I-A			
	Basal excretion	After control dose	Control dose excreted	Basal excretion	After test dose	Test dose excreted	Availability B_2 in I-A yeast
	mg/24 hr.	*mg/24 hr.*	%	*mg/24 hr.*	*mg/24 hr.*	%	%
F.F.	0.83	2.35	51	0.73	0.93	7	14
B.N.	0.77	2.33	52	0.70	1.06	14	27
M.M.	0.84	2.47	54	1.02	1.04	1	2
L.O.	0.88	3.19	77	1.02	1.06	2	3
D.O.	0.79	1.98	39	0.74	0.73	0	0
Average	0.82	2.46	55	0.84	0.96	5	9

A 150-gm dose of raw yeast I-A containing 2.6 mg of riboflavin released little or none of its riboflavin for absorption (table 2). The excretions ranged from 0 to 14% of the intake for the 5 subjects with an average of 5% while the urinary return from a control dose of pure riboflavin ranged from 39–77% with an average of 55%. It is not clear why the appreciable decrease in the urinary output of riboflavin on the ingestion of fresh yeast I-A recorded in figure 1 has not been observed subsequently in this or in further studies with fresh live yeasts (unpublished data) with the Melnick bioassay technic. In any event, the relatively small individual variations in riboflavin return in all experiments on live non-fortified yeasts are of little importance when this group as a

whole is compared with the output of subjects on a positive control dose or on live yeasts highly fortified with riboflavin. In these yeasts the vitamin addition presumably remains outside of the yeast cell inasmuch as it is returned in the urine so consistently. The details of this latter experiment will be reported elsewhere.

The riboflavin of dried dead yeast II-C, similar to I-C in all phases of manufacture except in thiamine content was found to be almost completely available (table 3). These results confirm those observed with yeast I-C.

TABLE 3

Availability to human subjects of riboflavin of dried yeast II–C with no viable cells.

SUBJECT	CONTROL PERIOD: 4.0 MG PURE RIBOFLAVIN IN SOLUTION			TEST PERIOD: 3.4 MG RIBOFLAVIN IN 82 GM DRIED YEAST II-C			
	Basal excretion	After control dose	Control dose excreted	Basal excretion	After test dose	Test dose excreted	Availability B_2 in II-C yeast
	mg/24 hr.	*mg/24 hr.*	%	*mg/24 hr.*	*mg/24 hr.*	%	%
D.H.	1.34	3.60	57	1.26	3.10	54	95
E.P.	0.69	2.81	53	0.75	2.59	54	102
R.N.	1.11	2.96	46	1.23	3.23	58	126
H.N.	0.77	3.06	57	1.17	2.78	47	82
Average	0.98	3.11	53	1.10	2.92	53	101

Further experiments with a commercial viable dried yeast also confirmed the results obtained with I-B. Yeast V-B, bought on the open market, was similar to yeast I-B in riboflavin content and viability, and was fed to 9 subjects, 5 women and 4 men. Urinary returns after the ingestion of 3 mg of riboflavin in the form of 63 gm of this yeast indicated that this riboflavin was only partially available as only 11–18% of the yeast test-dose was returned in the urine as compared to the 41–59% excretion of a control dose of pure riboflavin by these same subjects (table 4). This comparison indicates that not more than about 20–30% of the 3.0 mg of riboflavin in the

yeast was absorbed. These results support the hypothesis previously stated, that the tenacity of the living yeast cell is responsible for withholding the riboflavin from absorption.

TABLE 4

Availability to human subjects of riboflavin of retail viable dried yeast V–B.

SUBJECT	CONTROL PERIOD: 3.0 OR 4.0 MG PURE RIBOFLAVIN IN SOLUTION [1]			TEST PERIOD: 3.0 MG RIBOFLAVIN IN 63 GM RETAIL VIABLE DRIED YEAST V-B			
	Basal excretion	After control dose	Control dose excreted	Basal excretion	After test dose	Test dose excreted	Availability B_2 in V-B yeast
	mg/24 hr.	*mg/24 hr.*	%	*mg/24 hr.*	*mg/24 hr.*	%	%
D.H.[1] ♀	1.34 [1]	3.60 [1]	57	1.59	1.93	11	19
J.D.[1] ♀	1.13 [1]	3.41 [1]	57	0.73	1.17	15	26
B.S.[1] ♀	1.51 [1]	3.79 [1]	57	0.81	1.35	18	32
M.P. ♀	0.57	2.37	60	0.80	1.13	11	18
L.B. ♀	1.31	3.09	60	1.04	1.47	11	18
A.P. ♂	0.93	2.64	57	1.06	1.56	17	30
T.S. ♂	1.04	2.88	61	0.75	1.32	19	31
C.V. ♂	1.42	2.64	41	1.34	1.64	10	24
J.E. ♂	1.00	2.73	58	1.04	1.43	13	22
Average [2]			56			14	24

[1] A 4.0-mg control dose of riboflavin was substituted for 3.0 mg for these subjects.

[2] Averages for columns 2, 3, 5 and 6 are omitted because they are not really comparable.

SUMMARY

The degree of absorption of riboflavin of various yeasts from the digestive tract was judged by urinary excretions and, in some experiments, fecal outputs, in studies utilizing human bioassay methods.

There was little or no absorption of riboflavin from the fresh, raw yeast and only partial absorption from 2 dried yeasts which contained live cells. The destruction of the viability of the yeast in 3 samples resulted in release of the riboflavin rendering it available for absorption.

Fecal riboflavin eliminations were in a general reciprocal relationship to urinary excretions of the vitamin, indicating that when urinary excretions showed failure of absorption of the yeast riboflavin, fecal riboflavin was increased. Both

unabsorbed riboflavin and intestinal synthesis appeared to contribute to increases in fecal riboflavin.

ACKNOWLEDGMENTS

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THE AVAILABILITY OF VITAMINS FROM YEASTS

IV. THE INFLUENCE OF THE INGESTION OF FRESH AND DRIED BAKERS' YEASTS VARYING IN VIABILITY AND IN THIAMINE CONTENT ON THE AVAILABILITY OF THIAMINE TO HUMAN SUBJECTS [1]

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ONE FIGURE

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Human biological assays in this laboratory and elsewhere have been summarized (Parsons et al., '45b) and extended (Hochberg et al., '45) showing that bakers' compressed yeast, when fed fresh to human subjects is a poorly utilized source of thiamine as indicated by low urinary and high fecal thiamine eliminations. There was even some evidence (Parsons et al., '45b) that the ingestion of fresh yeast might be responsible for reducing the urinary thiamine output below that of a preceding basal period thus implicating yeast as a factor in reducing the availability of food thiamine. It was desired to explore this relationship further by lengthening the period of yeast ingestion and varying the dose.

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Procedures including air-drying, boiling and treatment with alcohol which reduced or destroyed viability of the yeast cell effected a marked improvement in the release of thiamine for absorption by animals (Walker and Nelson, '33; Parsons and Collord, '42; Parsons et al., '45a). Since this implied that the availability of yeast thiamine rests more directly on the vitality of the cell than on any other factor, studies were conducted to determine the availability of thiamine to human subjects from yeasts after drying processes in which the viability of the yeast cell was retained in some samples and entirely destroyed in others.

It was also observed that fresh yeasts of high thiamine content may release a varying portion of their thiamine for human absorption (Parsons et al., '45b). Hence this effect of increasing thiamine concentration upon availability of yeast thiamine was studied further.

EXPERIMENTAL

Determinations are reported of the urinary thiamine excretions of 15 subjects in a series of 16 diet studies in which thiamine supplements in the form of thiamine hydrochloride or yeast were added to a controlled basal diet. The 2 types of bioassay technic used and the details of the basal diet are described in the preceding paper (Price et al., '47). The method formerly in use in this laboratory was employed for the studies recorded in figure 1 and the bioassay of Melnick for those in tables 2, 3 and 4. That results with the latter method are satisfactorily reproducible in different laboratories was attested by the fact that the 2.0-mg supplement of pure thiamine hydrochloride fed to subjects in these experiments led to a 16.7 to 20% urinary return, thus falling well within the published range.

In addition to fresh yeast F and the 3 types of dried yeasts with varying degrees of viability (yeasts II-C, V-B and L-B) 2 others were used in these experiments, in which a second effect, that of increasing thiamine concentrations in the yeasts upon yeast thiamine availability, was studied. Yeast II-Z_3

and III-Z_3 were high vitamin yeasts containing 1.0 mg of thiamine per gm on a dry basis, or approximately 50–70 times the amount found in ordinary compressed bakers' yeast. They are of further interest since the thiamine content of the yeasts was attained by 2 different methods. The high content of yeast II-Z_3 was reached by synthesis and absorption of thiamine by the yeast itself during propagation in a nutrient medium. In the case of yeast III-Z_3 only a small percentage (approximately 2%) of the thiamine was attained by the yeast itself. The remainder of the thiamine was added in the form of thiamine hydrochloride to the thick suspension of resting

TABLE 1

Characterization of the baking yeasts tested for their influence on availability of thiamine and riboflavin.

IDENTITY OF THE YEASTS	CONTENT, DRY BASIS		REMARKS ON TYPE
	Riboflavin	Thiamine	
	μg/gm	*μg/gm*	
I–A [1]	55	19.5	Fresh compressed type. Live cells
I–A (boiled)	55	19.5	Same lot. Cells killed by heat.
I–B [1]	55	12.0	Same lot. Dried with retention of viability.
I–C [1]	55	11.8	Same lot. Dried with total loss of viability.
II–C [1]	47	65.5–74.3	Like I–C except high in thiamine acquired in growth.
II–Z_1 [1]		100	High thiamine acquired during growth by biosynthesis and absorption. Live cells.
II–Z_2 [1]		500	
II–Z_3 [1]	44	1000	
III–Z_1 [1]		100	High thiamine chiefly from fortification. Live cells.
III–Z_2 [1]		500	
III–Z_3 [1]	45	1000	
F [2]	55	18	Regular, fresh, for use by bakers. Live cells.
V–B [2]	48	10	Retail dry yeast for leavening. Live cells.
L–B [2]	146	68	Fortified retail dry yeast for leavening. Live cells.

[1] Special lots obtained directly from the manufacturer.

[2] Commercial products.

cells and thoroughly distributed by mechanical mixing before compression. These yeasts were fed to 5 subjects before each meal of the test day in doses equivalent to 5 mg of thiamine and urinary excretions of thiamine were compared with those obtained after subsequent test doses of 5 mg of thiamine hydrochloride. The other members of the II-Z and III-Z series in table 1 were used in *in vitro* experiments only.

Thiamine was determined by the thiochrome method of Hennessey ('42).

RESULTS

The implication given in an earlier study (Parsons et al., '45b) of an interference with absorption of food thiamine from the ingestion of fresh yeast was fully borne out by the

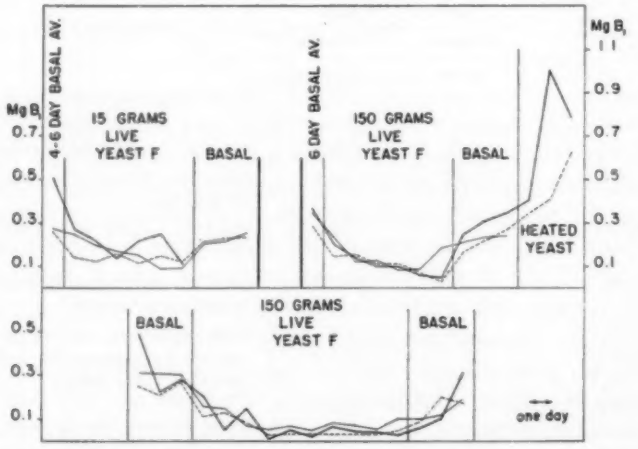

Fig. 1 The effect of ingestion of live yeast on the urinary output of thiamine.

feeding of 15 gm or 150 gm of yeast F for periods of 5 or 10 days. (See fig. 1. The averages of these data were published in a preliminary report by Ness et al., '46). The urinary output of thiamine fell below the basal level on the first day of yeast ingestion and continued to fall rapidly for about 4 days, reaching an average level of about 100 μg output for

the 15-gm yeast dose and 50 μg for the 150-gm dose; the minimum excretions reached as low as around 20 μg. Within the limits of the experiment, therefore, the greater the amount of yeast fed, the greater the apparent interference with absorption of food thiamine. Somewhat the same correlation was seen in the response to the removal of the live yeast from the diet: the longer the time on the larger dose of yeast, the slower the resumption of the previous output of urinary thiamine when the basal diet alone was again fed.

These results have a theoretical interest, in that this is a new example of interference with food thiamine to be added to others reported in the literature, notably the destruction of thiamine in the digestive tract by thiaminase from certain fishes and sea foods in the diet, the thiamine deficiency in cattle resulting from the ingestion of bracken and the antithiamine action of certain analogues of thiamine (Wright, '47, a review).

Practical applications of the results in regard to the dietary status of live yeast are obvious. Doses of yeast larger than those recommended by the manufacturer were deliberately included in this experiment inasmuch as it seemed probable that yeast intake by the public might include a considerable range of dosage in instances where fresh yeast was consumed with the notion of using it as a supposed therapeutic agent against furunculosis or acne rather than primarily as a vehicle for a given vitamin allowance.

The results in the feeding of 3 types of dried yeast in 2 of which the viability was retained and in the other, destroyed, indicated that drying as such had little or no relationship to the effect of the yeast on the absorption of thiamine from the human digestive tract, but a correlation was evident with respect to the vitality of the yeast cell after the drying process (table 2). In 8 of the 9 tests with subjects ingesting the thiamine supplement in the form of live, dried yeasts urinary outputs were maintained at levels no higher than those of the preceding basal period which would indicate no measurable availability of yeast thiamine; in fact, in 7 of the

9 tests, the subjects responded to the dose with a decrease from the previous basal urinary level. This has been shown to be characteristic of the response to fresh compressed yeast intake. Only 1 subject, D.H., exhibited an increase in urinary thiamine excretion from 1 of the 2 yeast supplements, an amount equivalent to 1% of the yeast thiamine intake; calculated from the percentage increment following the positive control dose of 2.0 mg of thiamine hydrochloride, the physio-

TABLE 2

Changes in urinary thiamine following ingestion of the vitamin in dried yeasts and in pure solution.

SUBJECT	PORTION OF TEST DOSE EXCRETED				SUBJECT	PORTION OF TEST DOSE EXCRETED	
	Control dose 2.0 mg thiamine		Increments or decrements due to doses live dried yeasts			Control dose 5.0 mg thiamine	Dead, dried yeast II–C 5 mg thiamine
			Yeast L-B	Yeast V-B			
	μg	%	μg	μg		%	%
B.S.	400	20.0	—9	—15	E.P.	19.3	21.3
D.H.	395	19.7	+21	—37	D.H	19.7	20.4
J.D.	330	16.5	—26	—75	H.N.	20.5	18.4
C.S.	425	21.3	—119		R.N.	20.9	23.0
Z.A.	360	18.0	0				
A.W.				—32			
Average		19.1				20.1	20.8

logical availability of this yeast to D.H. was roughly 7% which fell far short of the full availability claimed by the manufacturer for this type of yeast.

Regardless of the amount of live yeast ingested in these experiments none of the subjects experienced diarrhea. Laxation rate as measured by the appearance of carmine markers in fecal eliminations (Parsons et al., '44) was not uniformly affected by the ingestion of live yeast; evacuation time in those experiments ranged from 13 to 97 hours with no apparent correlation with the amount of urinary thiamine output.

In contrast to the results with live dried yeast, observation of 4 subjects ingesting 5.0 mg of thiamine in the form of a nutritional type of dried yeast, II-C, in which the yeast cells

were dead indicated full availability of the yeast thiamine. In all cases the increase in urinary thiamine output which followed the ingestion of this yeast fell within the range of from 19 to 23% excretion of yeast thiamine comparable to that obtained from the 5.0-mg control dose given these same subjects thus indicating that thiamine was fully available from this dead, dried yeast.

Data in tables 3 and 4 indicate the effect of increasing thiamine concentrations upon the availability of yeast thiamine. Yeast series II-Z and III-Z differed in that the content

TABLE 3

In vitro release of self-synthesized and mechanically added thiamine from yeasts.

IDENTITY OF YEASTS		THIAMINE CONTENT	THIAMINE FOUND PER ENTIRE SAMPLE					
			In solution		In yeast residue		Total	
		mg/dry gm	*mg/dry gm*	*%*	*mg/dry gm*	*%*	*mg/dry gm*	*%*
II–Z								
Thiamine synthesized or absorbed during propagation	II–Z_1	100	1.0	1.0	98	98	99	99
	II–Z_2	500	5.0	1.0	495	99	500	100
	II–Z_3	1000	40.0	4.0	940	94	980	98
III–Z								
90% or more of thiamine added after propagation	III–Z_1	100	43	43	65	65	108	108
	III–Z_2	500	330	66	170	34	500	100
	III–Z_3	1000	830	83	170	17	1000	100

of yeast series II-Z was attained by synthesis and absorption during propagation whereas in the case of III-Z thiamine was added mechanically to a suspension of mature yeast cells immediately before compression. As a precaution, yeast series III-Z was maintained at very cool temperatures throughout the fortification process and until the yeasts were tested *in vitro* or eaten by the subjects. By such a method of preparation it was believed that the greater part of the added thiamine might not be removed from the suspension medium by the live yeast cell.

In vitro studies (table 3) showed a more striking difference than did human bioassays (table 4) in the effect of the 2 methods of thiamine attainment upon its release by the yeast cell. Assays of thiamine released *in vitro* into supernatant solutions and that held by the yeast residue were conducted on both types of yeast with graduated thiamine content, following 2 minutes of shaking and 10 minutes of centrifugation

TABLE 4

Urinary excretion of thiamine following ingestion of vitamin in fresh yeasts II–Z_1 and III–Z_3 and in pure solution.

SUBJECT	CONTROL DOSE 5 MG THIAMINE HYDROCHLORIDE				YEAST II-Z_0 5 MG THIAMINE		YEAST III-Z_3 5 MG THIAMINE	
	Urinary thiamine/24 hrs.							
	Basal day	Test day	"Lag" day	Return of test dose [1]	Return of test dose [1]	Availability [1]	Return of test dose	Availability
	mg	*mg*	*mg*	%	%	%	%	%
SS	0.27	0.83	0.40	14.0	2.4	17.1	4.2	30.0
ED	0.26	1.10	0.39	19.4	2.0	10.3	2.8	14.4
PG	0.27	1.10	0.32	17.6	2.4	13.6	1.8	10.2
BS	0.29	1.15	0.44	20.2	5.8	28.7	4.6	22.7
JO	0.17	0.91	0.42	19.8	5.2	26.3	10.8	54.5
Average	0.25	1.02	0.39	18.2	3.5	19.2	4.8	26.4

[1] Urinary increment from dose = (μg urinary output of vitamin on test day — μg output on basal day) + (urinary output on "lag" day — output on basal day).

$$\text{Percentage return of vitamin in dose} = \frac{\text{urinary increment from dose}}{\text{amount of test dose}} \times 100.$$

$$\text{Percentage availability} = \frac{\text{percentage return of test dose}}{\text{percentage return of dose pure vitamin in solution}} \times 100.$$

in an acetate buffer solution at pH 4 similar to the one used by Hochberg et al. ('45). These *in vitro* results indicated that mechanically added thiamine was much less firmly held by the cell inasmuch as 42% was released into the supernatant fluid at the lowest level, increasing to 83% as the thiamine content of the yeast increased. In contrast the yeast which had attained its high thiamine content by synthesis and absorption during propagation released a maximum of only 4% of its thiamine into the supernatant layer. Human bioassays of thiamine availability from these 2 yeasts (table 4)

differed in the same direction as the supernatant concentrations, but the difference was much less than would have been expected from the *in vitro* results. The average 3.6% excretion of thiamine from yeast II-Z_3 and the 4.8% excretion of thiamine from yeast III-Z_3 are not far apart particularly in view of the large individual variations while both differ significantly from the average excretion of 18.1% following the ingestion of pure thiamine hydrochloride by the same 5 subjects. A similar divergence between the biological and *in vitro* release of thiamine from a high vitamin yeast has been reported by Hochberg et al. ('45). Whereas these workers found an *in vitro* release of 48–72% of thiamine in their high vitamin yeast, physiological availability was found to be only 17%.

The explanation of this evidence of differential release of yeast thiamine during *in vivo* and *in vitro* studies may be essentially the same as for the variation in the effects of different methods of drying of the yeast: i.e., a correlation with the activity of the yeast cell. It might be questioned whether under the less favorable conditions of the acetate-buffer medium in the *in vitro* experiments, metabolic activity of the yeast cell was sufficiently normal to permit a characteristic uptake of thiamine. On the other hand, a more favorable medium for active absorption of thiamine by the live yeast cell than the buffered solution used is apparently furnished either by fermenting wort, judged by the fate of thiamine in the brewing process (Levine, '41) or by the chyme of the upper digestive tract, as far as the evidence of reduced urinary return of thiamine when live yeast is eaten, is valid.

The mechanism for the inhibition of the availability of food thiamine is not known, but appears to be a competitive absorption of thiamine into the yeast cell in the digestive tract of the host. Other possibilities are being evaluated (Parsons et al., '47).

SUMMARY

Thiamine in an unfortified fresh bakers' yeast ingested by human subjects was unavailable for absorption. Yeasts high

in thiamine content, either from propagation or from fortification yielded only a small proportion of their thiamine for absorption; in the case of the highly fortified yeast a much larger proportion of thiamine was released *in vitro* in comparison.

The ingestion of a fresh unfortified bakers' yeast as a supplement to a weighed basal diet lowered the urinary output of thiamine and therefore apparently interfered with the absorption of food thiamine, this effect being greater on the larger yeast dose of 150 gm per day. The duration of the period of ingestion of fresh yeast affected the speed with which the previous level of urinary output of thiamine was again attained when yeast was removed from the diet, indicating a progressive depletion of body stores from ingestion of fresh yeast.

The drying of bakers' yeast did not of itself influence significantly the effect of the yeast on thiamine absorption; live dried yeast suitable for leavening also withheld its thiamine from absorption and interfered with the absorption of food thiamine judged by decreases in urinary output. On the other hand, bakers' yeast killed by commercial drying processes or by treatment in boiling water released its thiamine for absorption and offered no interference to absorption of food thiamine.

It is believed that live yeast cells in the digestive tract compete with the host for thiamine that is present.

ACKNOWLEDGMENTS

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A STUDY OF THE HEMORRHAGIC-KIDNEY SYNDROME OF CHOLINE DEFICIENCY: THE PROTECTIVE EFFECT OF STARCH

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Griffith and Wade ('39) observed a dramatic series of events occurring in young rats 6 to 8 days after they were placed on diets deficient in choline. Necrosis, congestion and sometimes hemorrhages involving the cortical portions of the kidneys occurred, producing swollen, tense, "hemorrhagic" kidneys. Hemorrhages also occurred less frequently in the eyes, and occasionally in other organs. The syndrome was fatal in a large percentage of the animals, presumably due principally to the renal insufficiency which ensued (Griffith and Mulford, '41b). However, the animals which survived the critical period of 2 to 4 days after onset of illness showed rapid recovery, even on the same deficient diet, and the hemorrhagic appearance of the kidneys disappeared entirely within a few days, in spite of the progression of the fatty changes in the livers (Griffith, '40b). The syndrome occurred most constantly in male animals under 30 days of age.

The addition of choline to the diet completely prevented the syndrome (Griffith and Wade, '39). Methionine (Griffith and Wade, '40) and betaine (Griffith and Mulford, '41a) were also effective, and it was presumed that this was explained by the utilization of methyl groups of these substances in the synthesis of choline by the process of transmethylation described by du Vigneaud et al. ('39, '41).

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Various vitamin B factors, and vitamins K, C, and P were ineffective against the lesions produced (Griffith and Mulford, '41b). The syndrome could be prevented by limitation of the food intake. Increasing the casein level of the diet to about 25% increased the incidence, perhaps due to the more rapid growth, but above this level there was a rapid decline in incidence (Griffith and Wade, '40), supposedly due to the increased amount of methionine (above that required as methionine and cystine) available for choline synthesis (Griffith, '41a). Cystine, cholesterol and fat increased the lesions (Griffith, '40a). Unilateral nephrectomy in adult rats on a choline-deficient diet caused the syndrome to develop in the remaining kidney during the period of hypertrophy (Handler, '46). Renal decapsulation or administration of atropine afforded protection in young rats, suggesting that the tubular necrosis is secondary to vascular changes, perhaps of neural origin (Dessau and Oleson, '47), but the effect of the procedures on food intake was not recorded.

During the course of an investigation of the liver and kidney injury produced in rats by diets containing pyridine (Baxter, '45; '46), it seemed desirable to use a diet low in choline and methionine but not so low that pathological changes would result, during the experimental period, from the diet alone. Diets containing 10–18% casein, with yeast, cod liver oil, corn starch, sucrose, lard and salt mixture, without added choline were used, and apparently were satisfactory. On these diets young rats somewhat above the most susceptible age were never observed to develop hemorrhagic kidneys. Later, however, when the starch was replaced by sucrose, some of the animals did develop hemorrhagic kidneys.

Griffith ('41b) stated, without presenting data, that starch prevented hemorrhagic kidneys, and Handler and Dubin ('46) and Handler ('46) apparently observed some degree of protection. The mechanism of this protective effect was not investigated by these authors, and no attempt was made to explain it.

In the present investigation, the effects of sucrose and corn starch were compared, in more highly purified diets than those used in the pyridine experiments, and when the incidence of illness and death due to hemorrhagic kidneys was found to be significantly lower with starch, efforts were made to determine the mechanism of this effect. There seemed to be at least 3 possible explanations: (1) a choline content of the starch sufficient to give some degree of protection, (2) the presence in the starch of 1 or more factors other than choline, capable of affording protection by forming choline or through some other action, and (3) an effect of starch itself in the intestinal tract, probably on the bacterial synthesis of choline or other substances.

EXPERIMENTAL

Animals

Twenty-one to 23-day-old rats (Sherman strain) were placed for several days on a stock diet and then transferred to experimental diets. Most of the animals in the various experiments weighed from 30 to 45 gm at the time the experimental diets were started. In each individual experiment, 6 to 8 animals of comparable ages and weights were run simultaneously on the different diets. Care was exercised to make comparisons only between similar groups. The animals were kept in individual cages with large-mesh screen bottoms. Approximately 250 rats were used.

Diets

The composition of the basal diet used in the experiments is shown in table 1. The diet was prepared with sucrose or corn starch as desired, and the various supplements added. Corn starch extracted by the procedures to be described, was used in some experiments. Corn starch heated in an oven at 120°C. for 4 hours, and potato starch [2] were each used in 1 experiment.

[2] Manischewitz.

All diets were given *ad libitum* and the amounts eaten recorded at intervals of 1 to 3 days.

TABLE 1

Composition of basal diet.

	%
Casein [1]	20
Hydrogenated vegetable oil	19
Corn oil	1
Sucrose or corn starch [2]	56
Salt mixture [3]	4

Supplements per Kilogram of Diet			
	mg		*mg*
Thiamine chloride	20	Para-amino-benzoic acid	10
Pyridoxine	20	Biotin	0.1
Riboflavin	30	Folic acid	0.2
Ca pantothenate	50	2-methyl-1,4-naphthoquinone	1.5
Nicotinic acid	200	*dl* α-tocopherol acetate	15.0
Inositol	500	Vitamin A concentrate	60,000 U.S.P. units
Ascorbic acid	500	Vitamin D concentrate	6,000 U.S.P. units

[1] Vitamin test casein.
[2] "Argo" corn starch, Corn Products Refining Co., Argo, Ill.
[3] Osborne and Mendel.

Choline determinations

Choline determinations were done by the reineckate method as modified by Glick ('44), with the following adaptions. Duplicate 25.0 gm samples of corn starch were mixed with equal amounts by weight of pulverized pumice stone, and 10.0 gm samples with twice the equivalent amounts of pumice. Each sample was placed in a 33 × 94 mm extraction thimble and extracted in a Soxhlet extractor connected with a 250 ml boiling flask, using a single 125 ml volume of methyl alcohol. The extractions were allowed to proceed for 24 to 36 hours. Liberation of bound choline was accomplished by hydrolysis with 50 ml of a saturated solution of barium hydroxide. Separation of the choline reineckate precipitates by filtration was performed in a cold-room. The precipitates were dissolved in acetone which was collected in colorimeter tubes, and

readings were made on a Klett-Summerson colorimeter. Known choline chloride samples were run simultaneously by adding them to 50 ml volumes of barium hydroxide and subjecting them to the same subsequent treatment as the unknowns.

Starch-extracts

Fifteen-hundred-gm samples of corn starch were extracted, twice by shaking with distilled water, 3 times by percolating with methyl alcohol, and once by percolating with ether. The solvents were separated by filtration and evaporated in a stream of warm air, leaving the extracted residues. Samples of some of the residues were taken for choline analyses. In 3 experiments, the combined residues from 1500-gm samples of starch were added to 500-gm lots of the sucrose-containing diet, and in a fourth experiment, the water and first alcohol residues, and the remaining residues, were added separately to similar amounts of the sucrose diet. The starch-extract diets, therefore, should have contained any extractable principle in about 5 times the concentration that was present in the starch-containing diet, assuming that extraction was complete and that none was destroyed in the process of extraction. The effects of the starch-extract-containing diets were compared with those of sucrose and starch diets.

Succinylsulfathiazole (SST)

Groups of animals were placed on diets containing sucrose, starch, starch plus 2% SST, and starch plus 2% SST plus 0.2% choline chloride, and the effects of these diets compared.

Examination of animals

Animals were usually observed daily, weighed, and examined for intraocular hemorrhages. Those which died during the experimental periods were autopsied and the kidneys, livers and other organs examined grossly. Microscopic sections of livers and kidneys were examined in a few cases.

Some of the surviving animals in the early experiments and all of them in the later experiments, were autopsied after about 10 days on the diets. The animals of 1 group on the choline-deficient sucrose diet were killed at intervals during the development of the renal lesions in order to observe the early changes, and a small group on the starch diet were continued on the diet for 4 months so that the chronic effects might be noted.

Examination of urine and feces

The gross characteristics of the urine and feces of all of the groups were noted, and the urine from a few animals was examined for albumin and blood. Groups of 4 adult rats each, were placed on diets containing starch, sucrose, and starch plus 2% SST, and after 4 days on the diets, collections of feces were started and continued for 10 days. Choline determinations were done on the specimens.

RESULTS

Comparison of effects of sucrose and starch diets

As summarized in table 2, the incidence of illness and death from hemorrhagic kidneys was considerably greater on the

TABLE 2 [1]

Comparison of effects of diets containing sucrose and starch.

DIET	NUMBER OF ANIMALS	ILL WITH LOSS OF WEIGHT [2]	DEAD WITH HEMORRHAGIC KIDNEYS	AVERAGE DAILY WT. GAIN FIRST 5 DAYS [3]
		%	%	gm.
Sucrose	50	86	62	2.0
Corn starch [4]	44	43	16	2.7

[1] This table includes some of the animals of other tables.

[2] In some of the early experiments, the surviving animals were not autopsied and the exact incidence of hemorrhagic kidneys was not known, but these figures represent the approximate incidence.

[3] In the occasional animal which became ill or lost weight during the fifth day, the average gain during the first 4 days was used in calculating these figures.

[4] In some experiments extracted corn starch was used, while in others starch was employed without extraction. The protective effect was not decreased by extraction.

sucrose diet than on the same diet containing corn starch. The animals usually became ill a day or 2 earlier on the sucrose diet. Food intake and growth were greater on the starch diet, so that the protection was not due to limitation of food intake.

Potato starch was used in place of corn starch in 1 experiment and seemed to be effective in preventing hemorrhagic kidneys. However, so much of the starch passed through the animals undigested, that growth was poor in spite of much larger food consumption than on the corn starch. With an equal food intake, the feces from a group of animals on potato starch weighed (after drying) 7 times as much as that from a group on corn starch. While the feces from animals on corn starch were grossly similar in appearance to that on sucrose, the feces on potato starch were much more bulky, lighter in color and similar to that described in cases of refection (Bliss, '36).

Choline content of corn starch

The corn starch was found to contain 13 to 15 mg choline chloride per 100 gm.[3] The amount of starch used per kilogram of diet mixture therefore contained about 80 mg of choline. It was found (table 3) that the addition of 100 mg of choline chloride per kilogram of diet did not significantly decrease the incidence of hemorrhagic kidneys, though this amount of choline did increase the rate of growth of the animals (compare with table 2), and the time of development of hemorrhagic kidneys was delayed by 1 to 2 days. Two hundred mg perhaps afforded about the same degree of protection as the starch, and an occasional animal was observed to develop the syndrome on the sucrose diet to which had been added 500 mg of choline chloride per kilogram.

Effects of corn-starch-extracts

After finding that the starch contained too little choline to account for its protective effect, it then seemed that there

[3] The choline determinations were done on the starch as it was used in the diets, without drying. There was a loss in weight of approximately 10% on drying in an oven at 100°C.

might be present other protective factors, which would be active when extracted and added to the sucrose diet.

To test this hypothesis, starch extracts were prepared as already described. Extraction with water produced a small amount of crystalline material, probably chiefly sugars. The water retained by the starch diluted in decreasing degree the alcohols used in successive extractions. A yellow pigment was removed principally by the first alcohol extraction; a brownish material, which was insoluble in ether and acetone and was probably zein, was found principally in the second one; the latter 2 contained considerable amounts of fatty substances. The ether extracts produced a pearly looking fatty material.

Choline determinations done on samples of the residues extracted from the 1500-gm lots of corn starch indicated that approximately one-third of the choline content of the starch had been extracted. This means that about 70 mg of choline were added to 500 gm of the sucrose diet by addition of the extracts.

In the first experiment comparing the effects of diets containing sucrose, starch, and sucrose plus starch-extract, the extract appeared to produce a questionably beneficial effect. However, in subsequent experiments using the extracts, the animals fared about the same or even worse than those on the sucrose diet alone (table 3), in spite of the fairly large choline content of the extracts. In one experiment using a limited number of animals, it seemed that heating the unextracted starch might have decreased its protective action. In order to rule out the destruction of an active principle by the process of extraction, 2 groups of rats were fed the diet containing extracted starch, and the survival was at least as great as with unextracted starch. It had to be concluded that the protective effect was due to an action of the starch as a whole, or possibly to substances not extracted under the conditions used.

TABLE 3

Effects of starch-extracts.

DIET	NUMBER OF ANIMALS	ILL WITH LOSS OF WEIGHT	DEAD WITH HEMORRHAGIC KIDNEYS	AVERAGE DAILY WT. GAIN FIRST 5 DAYS
		%	%	gm
Sucrose + combined extract	18	72	44	2.8
Sucrose + water-alcohol extract	6	83	67	2.5
Sucrose + alcohol-ether extract	6	67	50	2.8
Sucrose + 100 mg choline chloride per kilogram	18	78	44	2.7
Sucrose + 200 mg choline chloride per kilogram [1]				
Sucrose + 500 mg choline chloride per kilogram [1]				
Starch	18	39	11	2.9
Extracted starch	12	17	8	3.3
Heated starch	6	50	33	3.1
Potato starch	6	Some ill but not due to hemorrhagic kidneys	0	0.8

[1] The animals of these groups were not entirely comparable with those of the other groups.

Effects of succinylsulfathiazole (SST) on the protective action of starch

At this point in the investigation, it was considered probable that the effect of the starch was due to an influence on the production by bacteria in the intestine of choline or some factor necessary for the utilization of choline stores or capable of affording protection in some other manner. To investigate this possibility, starch-containing diets with added SST were used.

The addition of 2% SST to the diet did not cause the expected increase in incidence of hemorrhagic kidneys (see table 4). Actually the death rate was somewhat less than without the SST. Again the food intake and growth were better than in the control animals. It was noted that the caeca of the animals receiving SST were, in every case, much larger, at the end of the experimental period, than those of the ani-

mals receiving starch or sucrose diets without SST. This, however, was probably due chiefly to dilatation, and was not altered by additions of choline.

In the 2 later experiments utilizing SST, biotin and para-amino benzoic acid were omitted from the diet. This did not alter the results.

TABLE 4

Effects of succinylsulfathiazole (SST).

DIET	NUMBER OF ANIMALS	HEMORRHAGIC[1] KIDNEYS	DEAD WITH HEMORRHAGIC KIDNEYS	AVERAGE DAILY WT. GAIN FIRST 5 DAYS
		%	%	*gm*
Starch + 2% SST	14	43	7	2.4
Starch + 2% SST + 0.2% choline chloride	6	0	0	2.3
Starch	14	50	28	2.3
Sucrose	13	92	54	1.7

[1] The surviving animals were autopsied on the tenth day.

Choline content of the feces

Significant amounts of choline were not found in the feces on any of the choline-deficient diets.

Effect of choline on the consistency of the diet mixtures

The basal diet, particularly when made up with sucrose, was soft and sticky. When choline was added, the mixture immediately became much firmer and more granular. As little as 5–10 mg of choline chloride per 100 gm of diet produced a noticeable change in consistency. The mechanism of this effect was not extensively investigated but it seemed probable that it was due to a change in the physical state of the mixture not entirely explained by the hygroscopic effect of the choline chloride.

Pathological observations

Most of the animals which became ill on the choline-deficient diets, exhibited the renal lesions previously described by

others, consisting of intense vascular congestion and some hemorrhages, and necrosis of the renal tubules particularly in the cortex. Some of the animals which were killed while still apparently healthy exhibited hyperemia and congestion of the vessels in the capsule and adjacent tissue.

In the acute stages of illness, the urine usually contained albumin, and sometimes appeared red but was not found to contain blood. In the animals which survived the acute stages, the resolution of the lesions was much like that described by Christensen ('42). However one of a small group of animals maintained on the choline-deficient diet for 4 months, died, apparently of renal insufficiency, 3 weeks after being placed again on the stock diet, and the kidneys were found to be severely scarred.

The incidence of intraocular hemorrhages was a little more than one-tenth that of hemorrhagic kidneys. In most cases the hemorrhages appeared to originate near the periphery of the lens. Opthalmoscopic examination was attempted in a few animals where the hemorrhages were not so extensive as to make visualization impossible. No lesions to account for the hemorrhages were observed. Some animals which exhibited intraocular hemorrhages survived and were placed on the stock diet and observed for prolonged periods. In cases of massive hemorrhages, there was organization of the blood with fibrosis and loss of vision, while slight hemorrhages were resorbed with little visual impairment.

Most of the animals with hemorrhagic kidneys had livers which appeared slightly to markedly fatty. Two animals on the starch diet, which died presumably of hemorrhagic kidneys, were found on autopsy to have an acute focal necrosis of the liver, with grossly normal kidneys.

DISCUSSION

Fletcher, Best and Solandt ('35), using the method of acetylation and bioassay, reported a value for the choline content of corn starch higher than that found in the present study. It is possible that this discrepancy represented a real

difference in the choline content of the 2 starches. It is doubtful, however, that even the value reported by the investigators referred to, is sufficient to account for the anti-hemorrhagic-kidney effect of the starch. Furthermore, as has already been pointed out, the extracted starch, from which a significant portion of the choline had been removed, was just as effective as the unextracted starch.

Since others have experienced no difficulty in producing the hemorrhagic-kidney syndrome on diets containing 5 to 6% yeast, which must have contained at least 200 mg of choline per kg of diet according to the data of Engel ('42), it appeared unlikely from the beginning that the protective effect of starch was due entirely to choline. It was thought more probable that the starch contained other substances with labile methyl groups, not precipitated by reineckate in alkaline solution, or factors affording protection through some other mechanism, and that this study might provide an opportunity to isolate and identify the active substances. Engel ('42) observed that dried beef liver gave greater protection when fed with a choline-deficient diet than was accounted for on the basis of its choline content as determined chemically, and Lucas, Norris and Heuser ('46) reported that crude diets contained more "choline" by the microbiological method than by the reineckate determination. However, the inactivity of the starch-extracts, in spite of the fact that the extractable substances of the starch were not completely removed as judged by the choline content of the extracts, made the presence in starch of significant amounts of other protective factors unlikely. The diets containing the starch-extracts had a higher choline content, by a factor of approximately seven-fourths, and a much higher relative content of many of the other extractable substances, than the diets containing starch itself. The possibility remains, nevertheless, that the extracts may have contained some substances in lower proportion. More complete extraction of smaller amounts of starch, or of equal or greater amounts with elimination of the choline, would dispose of this uncertainty only if a positive result

were obtained in the form of greater protection by the extracts than accounted for on the basis of choline content, and this did not seem sufficiently likely to justify the laborious procedure.

The question of the effect of the starch on the intestinal synthesis of choline remained. du Vigneaud and associates ('39) demonstrated that labile methyl groups, which are an essential part of the choline molecule, cannot be synthesized in the body of most rats in necessary amounts, and constitute a dietary requirement, though an occasional rat may be able to grow on the methyl-free, homocystine-containing diet (with the carbohydrate in the form of sucrose). After feeding D_2O with a casein diet, it was shown (du Vigneaud, Simmonds, Chandler and Cohn, '45) that the methyl groups of choline isolated from the animals, contained a small amount of deuterium, indicating that some synthesis of methyl groups had occurred, probably in the intestine. Other investigators (Bennett, Medes and Toennies, '44) have observed more consistent growth on methyl-free diets, due, in Bennett's ('46) opinion, to a difference in the pre-experimental diet, and have obtained inhibition of growth by addition of succinylsulfathiazole to the diet (Bennett and Toennies, '46). However, since growth continued for a month after adding the SST, and was not resumed when the SST was removed without first returning the animals to the stock diet (Bennett, '46), it is not entirely clear what the effect of the SST was.

It has been shown that the bacterial flora of the intestine may be influenced by the composition of the diet (Herter and Kendall, '09), and that the amounts of vitamins produced by the intestinal bacteria may also be modified by changing the diet (Guerrant and Dutcher, '34). It seems, though, that if the protection afforded by starch against hemorrhagic kidneys was due to an increase in the synthesis of a protective factor in the intestine, that it would have been prevented by the SST. The results with SST did not rule out a decreased production of an aggravating factor as a result of substituting the starch for the sucrose, and it is possible that the choline-producing organisms were resistant to the SST in the con-

centration used, or that the 5- to 8-day period necessary for the development of the hemorrhagic syndrome was not long enough for the maximum effect of the SST.

Handler ('46) noted that hemorrhagic kidneys did not occur on diets with the carbohydrate in the form of lactose or galactose, apparently because of the general nutritional failure on these diets. This explanation was not applicable to the results obtained in this study with starch, for the nutritional state remained good.

The observation by others that the incidence of hemorrhagic kidneys in animals on choline-deficient diets is less when the animals are caged together than when kept in separate cages, suggested that choline determinations on the feces might provide evidence of intestinal synthesis. Biological assay of the feces for protective factors might have given more information than the chemical determinations.

The number of animals used was too small to permit definite conclusions regarding the apparent enhancement of the protective action of starch due to its extraction.

The acute focal necrosis of the liver and chronic renal injury of fatal degree, which were observed in a few animals in this study, have not been noted in similar studies by most investigators. Although the animals were apparently healthy in the pre-experimental periods, these lesions could not be attributed with certainty to choline deficiency. The intraocular hemorrhages bore at least a superficial resemblance to those of unknown etiology which occur fairly commonly in young men, and while it does not seem likely that the human disease is due to choline deficiency in most cases, the possibility that the defect may be related through other common factors to that in the experimental syndrome should be considered. The nature of the rather widespread vascular disturbance produced by choline deficiency, which may even be responsible for the renal necrosis, perhaps as a result of renal anoxia, has not been definitely determined or extensively investigated. The similarity of the renal lesions of the rats to those seen

in the human syndromes characterized by acute bilateral cortical necrosis, has been commented on by Christensen ('42).

SUMMARY

The incidence of illness and death due to the hemorrhagic-kidney syndrome in young rats on a choline-deficient diet, was significantly less when the diet contained corn starch, than when the carbohydrate was in the form of sucrose.

This effect of the starch was more than that produced by the addition to the sucrose diet, of more choline than was contained by the starch. Addition of increments of choline resulted in more rapid growth and a later appearance of hemorrhagic kidneys, before there was a significant decrease in the incidence of the syndrome.

Residues from extracts of the starch did not afford protection when added to the sucrose diet, and extracted starch was at least as effective as unextracted starch.

Addition of 2% succinylsulfathiazole to the starch-containing diet, did not diminish the protective action of the starch, and on none of the choline-free diets were significant amounts of choline found in the feces. The caeca of the animals receiving succinylsulfathiazole became markedly dilated.

In spite of the failure to obtain evidence of an increased bacterial synthesis of choline on the starch-containing diet, some effect within the intestinal tract appeared to be the most probable explanation of the protection afforded by starch, though the possibility of other protective factors in the starch was not entirely eliminated.

The lesions which were observed on the choline-deficient diets, and the significance of the vascular disturbance, were discussed.

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